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(54) Title: ARCHAEAL REPLICATION ACCESSORY FACTORS AND METHODS OF USE

(57) Abstract: This invention provides isolated polynucleotides that encode replication accessory factors. The invention also provides novel DNA replication accessory factors, which have been isolated and purified from the hyperthermophilic archaea *Pyrococcus furiosus*. The invention also provides various methods of enhancing a nucleic acid polymerase reaction comprising the addition of the replication accessory factors to the reaction. This invention further provides methods of synthesizing, amplifying, and mutagenizing nucleic acids of interest employing the replication accessory factors. This invention also provides kits comprising at least one of the replication accessory factors. This invention also provides kits useful for various methods that comprise at least one replication accessory factor.

Archaeal Replication Accessory Factors and Methods of Use

Related Application Information

This application claims the filing date benefit of U.S. Provisional Patent Application Serial No. 60/146,580, filed July 30, 1999, which is incorporated by reference in its entirety for any purpose.

Background And Summary Of The Invention

The invention relates to the field of replicating, amplifying, and sequencing nucleic acids. Further, this invention relates to novel proteins that enhance the activity of polymerases in polymerization reactions.

In vitro polymerization techniques have enormously benefited the fields of biotechnology and medicine. The ability to manipulate nucleic acids with polymerization reactions greatly facilitates techniques ranging from gene characterization and molecular cloning (including, but not limited to sequencing, mutagenesis, synthesis, labeling, and amplification of DNA), determining allelic variations, and detecting and screening of various diseases and conditions (e.g., hepatitis B).

An *in vitro* polymerization technique of great interest is the polymerase chain reaction (PCR). This method rapidly and exponentially replicates and amplifies nucleic acids of interest. PCR is performed by repeated cycles of denaturing a DNA template, usually by high temperatures, annealing opposing primers to the complementary DNA strands, and extending the annealed primers with a DNA polymerase. Multiple cycles of PCR result in an exponential amplification of the DNA template.

Unfortunately, PCR has limitations. These limitations range from: 1) the rate of nucleotide incorporation, 2) the fidelity of nucleotide incorporation, 3) the length of the molecule to be amplified, and 4) the specificity of the polymerase.

Various methods to improve PCR exist. One approach is to optimize the reaction conditions, e.g., such as the pH, dNTP concentrations, or reaction temperatures. Another approach is to add various chemical

compounds, e.g., formamide (Sarkar, G., et al. Nucl. Acids Res. 18: 7465 (1990)), tetramethyammonium chloride, and dimethyl sulfoxide (Chevet et al., Nucl. Acids Res. 23:3343-3344 (1995); Hung et al., Nucl. Acids Res. 18:4953 (1990)) to either increase the specificity of the PCR reaction and/or increase yield. Other attempts include adding various proteins, such as replication accessory factors. Replication accessory factors known to be involved in DNA replication have also increased yields and the specificity of PCR products. For example, *E. coli* single-stranded DNA binding proteins, such as ssb, have been used to increase the yield and specificity of primer extension reactions and PCR reactions (*see, e.g.*, U.S. Patent Nos. 5,449,603, and 5,534,407). Another protein, the gene 32 protein of phage T4, appears to improve the ability to amplify larger DNA fragments (Schwartz et al., Nucl. Acids Res. 18: 1079 (1990)).

An important modification that has enhanced the ease and specificity of PCR is the use of *Thermus aquaticus* (Taq) DNA polymerase in place of the Klenow fragment of *E. coli* DNA pol I (Saiki et al., Science 230: 1350-1354 (1988)). The use of this thermostable DNA polymerase obviates the need for repeated enzyme additions, permits elevated annealing and primer extension temperatures, and enhances specificity. Further, this modification has enhanced the specificity of binding between the primer and its template. Taq polymerase, however, has a fundamental drawback since it does not have 3' to 5' exonuclease activity. Thus, Taq polymerase cannot excise incorrect nucleotides incorporated into the ends of the amplified products. Due to this limitation, the fidelity of Taq-PCR reactions typically have suffered. Therefore, those in the field have searched for another thermostable polymerase that has 3' to 5' exonuclease activity.

Polymerases having 3' to 5' exonuclease activity have been found in archaebacteria (archaea). Archaea is a third kingdom of organisms, different from eukaryotes and bacteria (eubacteria). Many archaea are thermophilic bacteria-like organisms that can grow in extremely high temperatures, i.e.,

100°C. One such archaebacteria is *Pyrococcus furiosus* (Pfu). A monomeric polymerase from Pfu has been identified that has the desired 3' to 5' exonuclease activity and synthesizes nucleic acids of interest at high temperatures (Lundberg et al., Gene 108: 1-6 (1991); Cline et al., Nucl. Acids Res. 24: 3546-3551 (1996) (This polymerase is referred to as Pfu polymerase.)) A second DNA polymerase has been identified in *P. furiosus* which has two subunits (DP1/DP2) and is referred to as *P. furiosus* pol II. *See* References 1 and 15. This activity of this polymerase may also be enhanced by the addition of accessory factors to polymerization reactions.

Certain natural proteins exist in archaea, i.e., PEF (polymerase enhancing factors) that exhibit deoxyuracil triphosphatase (dUTPase) activity and that enhance the activity of Pfu polymerase (International Patent Application Publication No. WO 98/42860, published on October 1, 1998; U.S. Patent Application Nos. 08/822,774 and 08/957,709). The presence of deoxyuracil-containing DNA in a DNA polymerization reaction inhibits polymerase activity (Lasken et al., J. Biol. Chem. 271: 17692-17696 (1996)). Specifically, during the course of a normal PCR reaction, a dCTP may be deaminated into dUTP, thereby introducing a deoxyuridine into the newly synthesized DNA. When this newly synthesized DNA is used as an amplification template, the presence of the deoxyuridine inhibits the Pfu polymerase. The archaeal dUTPase (PEF) prevents dUTP incorporation and, thus, avoids the inhibition of the Pfu polymerase. Accordingly, the archaeal dUTPase optimizes the activity of Pfu polymerase.

According to certain embodiments, the invention provides methods of, and materials for, enhancing the polymerase activity of archaeal polymerases, including, without limitation, Pfu polymerase. Certain embodiments involve one or more archaeal replication accessory factors, that may be counterparts of certain eukaryotic proteins, e.g., helicase enzymes that unwind the DNA helix and, thereby, provide a single-stranded DNA template; single-stranded DNA binding proteins (e.g., RFA) that bind and

stabilize the resulting single-stranded DNA template; a "sliding clamp" protein (e.g., proliferating cell nuclear antigen or PCNA) that stabilizes the interaction between the polymerase and the primed single-stranded DNA template and that enhances synthesis of long DNA strands (also known as "processivity"); a "clamp-loading" protein complex (e.g., RFC) that assembles the PCNA protein; minichromosomal maintenance protein(s) (MCMs), possessing DNA binding activity, DNA-stimulated ATPase activity, and a 3'-5' helicase activity; CDC6, a protein that binds to the origin of replication through interactions with the origin replication complex (ORC) and facilitates the loading of MCM(s); and flap endonuclease-1 (FEN-1), a structure specific endonuclease and 5' exonuclease.

According to certain embodiments, the invention provides novel DNA replication accessory factors that have been isolated and purified from the hyperthermophilic archaea *Pyrococcus furiosus*. In certain embodiments, the isolated proteins are thermostable homologues of eukaryotic or prokaryotic DNA replication proteins PCNA, RF-C subunits, RFA, FEN-1, CDC6, and helicases, including but not limited to helicase 2-8, dna2, and MCM.

According to certain embodiments, this invention also involves isolated and purified polynucleotides that encode these novel replication accessory factors and analogs or degenerate variants of those polynucleotides.

In certain embodiments, the polynucleotide may be cDNA, genomic DNA, mRNA, partially or fully synthetic DNA, or plasmid DNA. The skilled artisan will understand that the complementary DNA and RNA sequences of any disclosed nucleic acid sequence is within the intended scope of the present invention.

According to certain embodiments, the invention includes vectors comprising a polynucleotide that encodes a replication accessory factor and host cells comprising such vectors. According to certain embodiments, the invention includes polypeptides expressed in those host cells. Further, this invention provides not only the host cells and their products, but also, the

methods of using such host cells to produce the polypeptides of interest. The isolated and purified polypeptides of the invention, as well as recombinantly produced polypeptides are within the scope of the invention.

According to certain embodiments, the invention includes methods of enhancing a nucleic acid polymerase reaction comprising the addition of one or more of the replication accessory factors to the reaction.

In certain embodiments, only one archaeal replication accessory factor will be added into the nucleic acid polymerase reaction. In other embodiments, a combination of factors may be added.

In certain embodiments, an archaeal dUTPase may be combined with one or more of those replication accessory factors to further enhance the polymerase reaction.

According to certain embodiments, compositions for enhancing nucleic acid polymerase reactions are provided. These compositions comprise archaeal polypeptides, either alone or in combination with other archaeal polypeptides, including, for example, PCNA, RFC, RFC-P55, RFC-P38, RFA, MCM, CDC6, FEN-1, ligase, dUTPase, helicases 2-8, and helicase dna2. In certain embodiments archaeal polypeptides are from or homologous to a polypeptide found in members of the *Pyrococcus* or *Thermococcus* genera. In other embodiments, the polypeptides are from or homologous to a polypeptide found in *Pyrococcus furiosus*. In certain embodiments, the compositions further comprise one or more polymerases, including, for example, thermostable polymerases and polymerases that lack 3' to 5' exonuclease activity. Methods for enhancing nucleic acid polymerase reactions comprising these novel compositions are also provided.

In certain embodiments, this invention also provides methods of synthesizing nucleic acids comprising employing an archaeal polymerase and an archaeal replication accessory factor(s).

According to certain embodiments, the invention includes methods of amplifying, mutagenizing, or labeling nucleic acids of interest comprising

employing an archaeal polymerase and an archaeal replication accessory factor(s).

In certain embodiments of the inventive methods, the archaeal polymerase is Pfu polymerase. In certain embodiments of those methods, the archaeal polymerase is combined with another polymerase, such as Taq, Stoffel, Tfl, Tru, Tca, Tfil, Tbr, or Tth polymerase. In other embodiments of these methods, an archaeal dUTPase may also be included to enhance polymerase activity.

In certain embodiments of the inventive methods, the archaeal polymerase is *P. furiosus* pol II polymerase. In other embodiments, *P. furiosus* pol II polymerase is combined with a second polymerase. In certain embodiments the second polymerase lacks a 3' to 5' exonuclease activity, for example, without limitation, Taq, Stoffel, Tth, Tfl, Tru, Tfil, Tca, or Tbr polymerase.

In certain embodiments, this invention also provides a kit used in the practice of the above-described methods.

In certain embodiments, this invention also provides a kit comprising an archaeal polymerase and at least one archaeal replication accessory factor.

In certain embodiments, those kits would also comprise an archaeal dUTPase and possibly, another polymerase, such as Taq.

While compositions of the present invention are particularly useful for PCR applications, such compositions have utility for nucleic acid polymerase reactions in general. For example, without limitation, synthesizing, labeling, amplifying, or mutagenizing nucleic acids. Accordingly, the present invention is not intended to be limited to PCR applications.

Brief Description of the Drawings

The abbreviations used herein for amino acids in the predicted amino acid (or translated protein) sequences, which are single letter, and the nucleic

acids are those conventionally used, as in Stryer et al., Biochemistry, 3rd ed., W.H. Freeman, N.Y. (1988) at the back cover.

Figure 1 illustrates the identification of native PCNA in heparin sepharose fractions. Nucleotide incorporation was measured in the absence of salt to detect polymerase activity ("Pol") or in the presence of NaCl + Pfu DNA polymerase to detect PCNA.

Figure 2 illustrates the identification of native PCNA activity in SDS-PAGE gel slices. An active heparin sepharose fraction was electrophoresed on an SDS-PAGE gel, and slices of the gel were excised and the proteins eluted. The presence of PCNA or polymerase activity was determined as described above in Figure 1 and in the Detailed Description of Embodiments of the Invention.

Figure 3 illustrates the DNA sequence of PCNA.

Figure 4 illustrates the predicted amino acid sequence of PCNA.

Figure 5 illustrates that PCNA enhances the processivity of Pfu DNA polymerase. A 5'-radiolabelled 38 bp oligonucleotide was annealed to single-stranded M13. The template was incubated at 72°C in the presence of cloned Pfu PCR buffer, dNTPs, and either cloned Pfu DNA polymerase or exo$^-$ Pfu DNA polymerase. To certain reactions, ~0.1 or 10 fmoles of PCNA was added. Reactions were allowed to proceed for 1, 5, 10, or 30 minutes, and then stopped in loading buffer. The extension products were electrophoresed on CastAway® prepoured 6% (7M urea) gels, and the gels were dried and visualized by autoradiography. The length of the fully extended product is approximately 7 kb.

Figure 6 illustrates the stimulation of TaqPlus® Long DNA polymerase blend (Stratagene) with PCNA. A 23 kb fragment was amplified from genomic DNA using 5U TaqPlus® Long polymerase blend,

in the presence of native PEF, a no KCl buffer, and varying amounts of PCNA.

Figure 7 illustrates the stimulation of TaqPlus® Long DNA polymerase blend with PCNA. A 30 kb fragment was amplified from genomic DNA using 5U TaqPlus® Long, in the presence of native PEF, a no KCl buffer, and varying amounts of PCNA.

Figure 8 illustrates the DNA sequence of genomic RFC clones. Genomic sequences encoding the P38 and P55 subunits are located in tandem, respectively. The sequence encoding P38 contains an intein. As used herein, the term "intein" includes, but is not limited to protein splicing elements. These elements are involved in the post-translational processing of pre-proteins. The coding regions of the P38 and P55 subunits are bracketed [ ]. The intein sequence is enclosed in parentheses ( ).

Figure 9 illustrates the predicted amino acid sequence of the genomic RFC clone. The sequence encoding P38 and P55, respectively, are enclosed in parentheses ( ), while the sequence of the intein is bracketed [ ]. The * indicates a stop codon.

Figure 10 illustrates the predicted amino acid sequence of recombinant P55 clone.

Figure 11 illustrates the predicted amino acid sequence of recombinant P38 clone.

Figure 12 illustrates a Western blot of immunoaffinity purified native RFC complex using anti-P38 IgG (panel A) or anti-P55 IgG (panel B). Immunoaffinity purification was carried out using rabbit anti P55 IgG as the capture reagent. Fractions are labeled as follows: +, positive control; 0, wash. F20-F34 refer to fractions eluted at pH 2.8 from the column.

Figure 13 illustrates a protein gel of immunoaffinity purified native RFC complex. Immunoaffinity purification was carried out using rabbit

anti-P55 (-P55) IgG as the capture reagent. Fractions are labeled as follows: +, positive P38 control; -P38 (unrelated expt.) or -P55 column washes (present expt.). F18-F23 refer to fractions eluted at pH 2.8 from the column.

Figure 14 illustrates the ATPase activity of native and recombinant RFC. Positions on the TLC plate containing the released radioactive phosphate were excised and counted in a scintillation counter.

Figures 15 illustrates that native clamp loader further stimulates primer extension by Pfu in the presence of PCNA. Primer extension reactions were carried out as described in the Detailed Description of Embodiments of the Invention.

Figure 16 illustrates a cDNA sequence of a clone expressing RFA. Nucleotide 7 is annotated ($_7$G).

Figure 17 illustrates the predicted amino acid sequence of RFA. The theoretical molecular weight is 41.3 kDa. The native protein may start at the third amino acid, methionine (annotated $_3$M). The four amino acids shown in bold and underlined identify a 4-cysteine-type zinc finger motif ($X_3CX_{2-4}CX_{12-15}CX_2C$; H is an acceptable substitute for C).

Figure 18 illustrates a gel shift assay that demonstrates single-stranded DNA binding activity of *P. furiosus* RFA. 50 ng of a 38-mer oligo was incubated with *E. coli* SSB (lane 1), water (lane 2), or *P. furiosus* RFA (lanes 3-7) in TE buffer (lanes 1-3), 1x cloned Pfu buffer (lane 4), 50 mM Tris pH 8.5, 25 mM KCl, 2 mM $MgCl_2$ (lane 5), 50 mM Tris pH 8.5, 25 mM KCl, 5 mM $MgCl_2$ (lane 6), or 50 mM Tris pH 8.5, 25 mM KCl, 2 mM $ZnCl_2$ (lane 7). Samples were incubated at 95°C for 10 minutes, followed by 72 °C for 2 minutes prior to loading on a 4-20% acrylamide gradient

gel in 1x TBE buffer. Bands were visualized by SYBR green staining and UV illumination

Figure 19 illustrates an increase in amplification specificity with RFA using cloned Pfu + PEF (Pfu Turbo™ DNA polymerase (Stratagene)) (5.2 kb system).

Figure 20 illustrates an increase in product yield using RFA in combination with cloned Pfu Turbo™ DNA polymerase (2.1 kb system).

Figure 21 illustrates an increase in yield and amplification specificity with RFA and *E. coli* SSB using *Taq* and *Pfu* DNA polymerases (0.5 kb system).

Figure 22 illustrates the DNA sequence of recombinant helicase 2. This helicase has demonstrated PCR enhancing activity.

Figure 23 illustrates the DNA sequence of recombinant helicase 3.

Figure 24 illustrates the DNA sequence of recombinant helicase 4.

Figure 25 illustrates the DNA sequence of recombinant helicase 5.

Figure 26 illustrates the DNA sequence of recombinant helicase 6.

Figure 27 illustrates the DNA sequence of recombinant helicase 7.

Figure 28 illustrates the DNA sequence of recombinant helicase dna2. This helicase has demonstrated PCR enhancing activity.

Figure 29 illustrates the predicted amino acid sequence for helicase 2. The theoretical molecular weight is 87.9 kDa + 4.0 kDa (CBP affinity tag).

Figure 30 illustrates the predicted amino acid sequence for helicase 3. The theoretical molecular weight is 100.0 kDa + 4.0 kDa (CBP affinity tag).

Figure 31 illustrates the predicted amino acid sequence for helicase 4. The theoretical molecular weight is 105.0 kDa + 4.0 kDa (CBP affinity tag).

Figure 32 illustrates the predicted amino acid sequence for helicase 5. The theoretical molecular weight is 86.8 kDa + 4.0 kDa (CBP affinity tag).

Figure 33 illustrates the predicted amino acid sequence for helicase 6. The theoretical molecular weight is 105kDa + 4.0 kDa (CBP affinity tag).

Figure 34 illustrates the predicted amino acid sequence for helicase 7. The theoretical molecular weight is 126.0 kDa + 4.0 kDa (CBP affinity tag).

Figure 35 illustrates the predicted amino acid sequence for helicase dna2. The theoretical molecular weight is 74.6 + 4.0 kDa (CBP affinity tag).

Figure 36 illustrates the ATPase activity of helicases produced by phage induction.

1 l of Pfu helicases 3, 4, 5, 6, 7, and 8 (lanes 1-6 respectively), 0.8 units of porcine ATPase (9) or water (10) were incubated with 1 l of 4.5 M ATP and 1 Ci of gamma labeled ^{33}P ATP in 1x Optiprime buffer #3 (10mM Tris-HCl (pH 8.3), 3.5 mM $MgCl_2$, 75 mM KCl). The sples were incubated at 72°C for 20 minutes before being spotted on PEI cellulose F. The samples were allowed to dry before the PEI cellulose was placed in a shallow reservoir of 0.4 M NaH_2PO_4. The liquid front was allowed to migrate 5 cm before being removed from the liquid and dried. The samples were exposed to x-ray film for one hour.

Figure 37 illustrates the ATPase activity of helicases produced by IPTG induction of bacterial cultures. 1 microliter of an old lot or new lot of Pfu dna2-like helicase (lanes 1 and 2, respectively), Pfu helicase 2, 3, 4, 5 and 7 (lanes 3-7), water (8), or 0.8 units of porcine ATPase (9) were incubated with 1 μl of 4.5 micromolar ATP and 1 microCurie of gamma labeled ^{33}P ATP in 1x

Optiprime buffer #3 (10mM Tris-HCl (pH 8.3), 3.5 mM $MgCl_2$, 75 mM KCl). The samples were incubated at 72°C for 20 minutes before being spotted on PEI cellulose F. After drying, the PEI cellulose was placed in a shallow reservoir of 0.4 M NaH_2PO_4. The liquid front was allowed to migrate 4 cm before being removed from the liquid and dried. The samples were exposed to x-ray film for one hour.

Figure 38 illustrates the helicase displacement of bound oligos. Radioactively labeled oligonucleotides with a 3' overhang (A) or a 5' overhang (B) were annealed to M13mp18. The reactions were incubated with 0.5 μl of putative Pfu helicases 3-7 and Pfu helicase dna2 in 50 mM Tris pH 8.5, 25 mM KCl, 5 mM $MgCl_2$ and 5 mM ATP for 30 minutes at 55°C. 1 μl of Pfu helicase 2 was used in an identical reaction. The positive control was generated by thermally melting the annealed oligo prior to loading. The negative control was incubated with water. The samples were run on 4-20% gradient acrylamide gels in 1xTBE. The gels were dried and exposed to x-ray film.

Figure 39 illustrates the enhancement of *Pfu* polymerase processivity with *Pfu* PCNA and RF-C.

Figure 40 illustrates the DNA sequence of recombinant helicase 8. Molecular weight is 82.6 kDa + 4 kDa CBP tag.

Figure 41 illustrates the predicted amino acid sequence of recombinant helicase 8.

Figure 42 illustrates the stimulation of nucleotide incorporation by Pfu and P. furiosus pol II DNA polymerases using PCNA. Primer extension reactions were performed at 66-99°C using primed single-stranded M13 DNA. Optimal activity was observed at or above 80°C in the presence of PCNA (no measurements

carried out between 80 and 95°C), while reduced activity was observed above 72°C in the absence of PCNA.

Figure 43 illustrates the DNA sequence of *Pfu* genomic helicase 8.

Figure 44 illustrates the predicted amino acid sequence of *Pfu* genomic helicase 8. The theoretical molecular weight is 82.6 kDa + 4 kDa tag.

Figure 45 illustrates the DNA sequence of *Pfu mcm* gene with the intein present. The underlined sequence corresponds to the oligonucleotide primer used in sequencing to confirm deletion of the intein and proper joining of the junction.

Figure 46 illustrates the predicted amino acid sequence of the *Pfu* MCM polypeptide with the intein present. The theoretical molecular weight is 118 kDa.

Figure 47 illustrates the predicted amino acid sequence of the *Pfu* MCM polypeptide with the intein removed. The theoretical molecular weight is 78.6 kDa.

Figure 48 illustrates the DNA sequence of the *Pfu cdc6* gene.

Figure 49 illustrates the predicted amino acid sequence of the *Pfu* CDC6 polypeptide. The theoretical molecular weight is 48.3 kDa.

Figure 50 illustrates FEN-1 Enhancement of a TaqPlus Long (TPL) PCR reaction containing *Pfu* PCNA. A 23kb -globin target was amplified from human genomic DNA with TaqPlus Long DNA polymerase in the absence (0 PCNA) or presence of 2 different amounts of Pfu PCNA (47.7 pM and 4.77 nM) and varying concentrations of Pfu FEN-1 (lanes 1-6 correspond to 0, 11.9 pM, 23.7 pM, 119 pM, 1.19 nM, and 11.9 nM, respectively).

Figure 51 illustrates FEN-1 Enhancement of a Pfu PCNA stimulated PfuTurbo PCR reaction. A 17 kb -globin target was amplified from human genomic DNA with Pfu Turbo DNA polymerase in the absence (0) of PCNA or FEN-1, the presence of PCNA

alone (131 pM), or the presence of PCNA and FEN-1 (131 pM PCNA, 774 pM FEN-1). Amplifications were carried out in the presence of a thermostable topoisomerase (ThermoFidelase; Chemicon). Either no (0) topoisomerase was added to the reaction, or 1 l of undiluted (1) or diluted (1/5, 1/10, or 1/25) topoisomerase was added to the reaction.

Figure 52 illustrates FEN-1 Enhancement of a Pfu PCNA stimulated PfuTurbo PCR. A 17 kb -globin target was amplified from human genomic DNA with Pfu Turbo DNA polymerase in the absence (0) or presence (95 pM) of PCNA and varying concentrations of FEN-1 (lanes 1-6 correspond to 0, 11.9 pM, 23.7 pM, 119 pM, or 1.19 nM, respectively).

Figure 53 illustrates the enhancement of a PCR reaction containing either MCM or MCM and CDC6. A 23kb genomic target was amplified with TaqPlus Long DNA polymerase in the absence (left side) of presence (right side) of CDC6 and the following amounts of P. furiosus MCM : none (0) or 1ul of MCM diluted 1/1000, 1/500, or 1/100.

Figure 54 illustrates a gel shift assay using reconstituted RFA. Lane 1 comprises the 60-mer oligo template and water (negative control). Lane 2 comprises the 60-mer oligo template and 1 g *E. coli* SSB (positive control). Lane 3 comprises the 60-mer oligo template and approximately 1 g reconstituted Pfu RFA-CBP. Lane 4 comprises the 60-mer oligo template and approximately 5 g reconstituted Pfu RFA-CBP.

Figure 55 illustrates the Pfu MCM DNA sequence with the intein sequence removed.

Detailed Description of Embodiments of the Invention

The invention provides for isolated and purified polynucleotides that encode novel DNA replication accessory factors from archaeabacteria. In certain embodiments, the replication accessory factors or polypeptides are from *Pyrococcus* species or the closely related archaeal species *Thermococcus*. These replication accessory factors may be thermostable homologs of the eukaryotic DNA replication proteins PCNA, RFC subunits, RFA, CDC6, FEN-1, and helicases, including without limitation, MCM. By thermostable is meant that the polypeptide has a biological half-life of at least 30 minutes at 50° C.

As used herein "isolated and purified polynucleotide" is a nucleic acid, which is substantially separate from at least one other nucleic acid sequence that naturally accompanies the native polynucleotide.

These polynucleotides include RNA, cDNA, genomic DNA, synthetic forms, e.g., oligonucleotides, antisense and sense strands, and may also include chemically or biochemically modified nucleotides, e.g., mutated nucleotides or cys-labeled nucleotides. The skilled artisan will understand that the term genomic DNA may include not only the coding region or exons, but also promoters, enhancers, introns, and other regulatory elements. Such regions are generally known in the art and can be identified using methods known to the skilled artisan. Recombinant polynucleotides, generated, for example, without limitation, recombinant technology and molecular giological techniques, are also provided. Also within the scope of the invention are fully or partially synthetic polynucleotide sequences, for example, without limitation, generated by solid phase synthetic processes. *See, e.g.,* Current Protocols in Molecular Biology, including supplements through July 2000, John Wiley & Sons; Current Protocols in Nucleic Acid Chemistry, 2000, John Wiley & Sons.

Although polynucleotides having naturally occurring sequences may be employed, such polynucleotides may be altered, e.g., by deletion,

substitution, or insertion. One skilled in the art will know appropriate changes in the sequence that will encode proteins that retain biological activity. In certain preferred embodiments, polynucleotides may be changed to encode different conservative amino acid substitutions. Conservative amino acid substitutions include, but are not limited to, a change in which a given amino acid may be replaced, for example, by a residue having similar physiochemical or biochemical characteristics. Examples of such conservative substitutions include, but are not limited to, substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another; substitutions of one polar residue for another, such as between Lys and Arg, Glu and Asp, or Gln and Asn; or substitutions of one aromatic residue for another, such as Phe, Trp, or Tyr for one another. Other conservative substitutions, *e.g.*, involving substitutions of entire regions having similar hydrophobicity characteristics, are well known. See Biochemistry: A Problems Approach, (Wood, W.B., Wilson, J.H., Benbow, R.M., and Hood, L.E., eds.) Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA (1981), page 14-15.

cDNA or genomic libraries of various types may be screened as natural sources of the polynucleotides of the present invention, or such nucleic acids may be provided by amplification of sequences that exist in genomic DNA or other natural sources, e.g., by PCR. See, e.g., PCR Protocols: A Guide to Methods and Application, Innis, M., et al., eds., Academic Press: San Diego (1990). Genomic polynucleotides encoding the archaeal replication accessory factors may contain additional non-coding bases, or inteins, and one skilled in the art would know how to obtain such polynucleotides. One way to obtain genomic DNA sequences is by probing a genomic library with all or part of a known DNA sequence. The obtained genomic DNA sequence should encode functional proteins.

In certain embodiments of this invention, the nucleic acid sequences of the isolated polynucleotides encoding the replication accessory factors have

been obtained and may be used for various purposes. In certain embodiments, the invention includes isolated and purified polynucleotides that encode the following: archaeal PCNA, archaeal RFC subunit P38 protein, archaeal RFC subunit P55, archaeal RFC subunit P98, archaeal RFA, archaeal CDC6, archaeal FEN-1, and various archaeal helicases. According to certain embodiments, the invention includes nine different helicases that exist in Pfu, i.e., helicase 2 to 8, MCM, and helicase dna2. Homologous polynucleotide or polypeptide sequences from other members of the Pyrococcus species and Thermococcus species are also within the scope of the invention.

As used herein, the term "PCNA" may also be referred to as a "clamp" or a "sliding clamp" protein, in view of its role in clamping the DNA polymerase to the DNA template in eukaryotes.

In certain embodiments, the term "RFC subunits" includes, but is not limited to, proteins of about 55 kilodaltons (kDa) and about 38 kDa in molecular weight or subunits having the amino acid sequence set forth in Figure 10 and Figure 11, respectively. These subunits are referred to herein as "P55" and "P38." These subunits are part of a complex having one large subunit and at least one small subunit. P55 is considered a large subunit and P38 a small subunit. The RFC P98 subunit comprises the P38 subunit and the intein sequences that are removed from the functional form of the P38 protein.

This invention further provides for isolated and purified polynucleotides that encode amino acid sequences for various replication accessory factors, such as an archaeal PCNA, archaeal RFC subunit P38 protein, archaeal RFC subunit P55, archaeal RFA, archaeal CDC6, archaeal FEN-1, and various archaeal helicases, including helicase 2-8, helicase dna2, and MCM.

The polynucleotides described herein also include nucleic acid sequences that encode for polypeptide analogs or derivatives of the various archaeal polypeptides, including replication accessory factors, which differ

from naturally-occurring forms, e.g., deletion analogs that contain less than all of the amino acids of the naturally-occurring forms, substitution analogs that have one or more amino acids replaced by other residues, and addition analogs that have one or more amino acids added to the naturally-occurring sequence. These various analogs share some or all of the biological properties of the archaeal polypeptide factors. As noted above, one skilled in the art will be able to design suitable analogs. In certain preferred embodiments, conservative amino acid substitutions will be made. In certain embodiments, the analogs will be 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or homologous to the naturally-occurring sequence.

Percent identity involves the relatedness between amino acid or nucleic acid sequences. One determines the percent of identical matches between two or more sequences with gap alignments that are addressed by a particular method. The percent identity may be determined by visual inspection and/or mathematical calculation. Alternatively, the percent identity of two nucleic acid sequences can be determined by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res. 14*:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Other programs used by one skilled in the art of sequence comparison may also be used.

In certain embodiments, polynucleotides may be those that hybridize under moderately or highly stringent conditions to the complement of

naturally-occurring encoding nucleic acids or to nucleic acids that encode proteins having naturally-occurring amino acid sequences. As used herein, conditions of moderate stringency can be readily determined by those having ordinary skill in the art based on, for example, the length of the DNA. The basic conditions are set forth by Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989), and include use of a prewashing solution for the nitrocellulose filters 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization conditions of about 50% formamide, 6X SSC at about 42°C (or other similar hybridization solution, such as Stark's solution, in about 50% formamide at about 42°C), and washing conditions of about 60°C, 0.5X SSC, 0.1% SDS. Conditions of high stringency can also be readily determined by the skilled artisan based on, for example, the length of the DNA. Generally, such conditions are defined as hybridization conditions as above, and with washing at approximately 68°C, 0.2X SSC, 0.1% SDS. The skilled artisan will recognize that the temperature and wash solution salt concentration can be adjusted as necessary according to factors such as the length of the probe.

In certain embodiments, polynucleotides may have sequences different from the naturally-occurring nucleic acid sequence in view of the redundancy (degeneracy) in the genetic code, especially if the amino acid sequences are known. The skilled artisan will understand that many amino acids are encoded by two or more different codons and that a polynucleotide containing these degenerate codons, while differing from the naturally-occurring nucleic acid sequence, still encodes the same amino acid sequence. Additionally, it may be desirable to introduce various codon substitutions into a polynucleotide sequence in order to create restriction sites or to optimize expression in a particular system. Such degenerate variants and substituted polynucleotides are within the scope of the present invention.

The polynucleotides used in this invention will usually comprise at least about 15 nucleotides. In certain embodiments, the number of nucleotides is the minimal length required to express a biologically active replication accessory factor or, to probe for nucleic acid sequences encoding a replication accessory factor, or for nucleic acid priming. Such minimal lengths can be readily determined by the skilled artisan using conventional molecular biology techniques or those disclosed herein.

Techniques for manipulating polynucleotides are described generally in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, (Cold Spring Harbor Laboratory Press 1989). Reagents useful in applying such techniques, e.g., restriction enzymes, are widely known in the art and commercially available from vendors such as Stratagene (La Jolla, CA).

These polynucleotides may be used as nucleic acid probes and primers. Such probes and primers would be useful in screening for other archaeal replication accessory factors or screening other species for homologous replication accessory factors. The probe or primer may comprise an isolated nucleic acid, and may include a detectable label, such as a reporter molecule.

In certain embodiments of this invention, one may also want to generate viral or plasmid DNA vectors using the polynucleotides disclosed herein. The contemplated vectors include various viral vectors. Some commonly used examples are, but are not limited to, plasmids, bacteriophages, retroviruses, baculovirus, and adenovirus. Such vectors may be coupled with nucleic acids that encode an origin of replication (ORI) or autonomously replicating sequence (ARS), expression control sequences, e.g., promoter and enhancer sequences, and protein processing information sites, such as RNA splice sites, polyadenylation sites, ribosome-binding sites, and mRNA stabilizing sequences. Such vectors and methods for constructing them are well known in the art. See, e.g., Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, Cold Spring Harbor Laboratory

Press, (1989); Pouwels et al., Cloning Vectors: A Laboratory Manual, Elsevier, New York, (1985); "Gene Expression Technology" Methods in Enzymology, v. 185, D.V. Goeddel, ed. Academic Press Inc., San Diego, CA (1990); and "Viral Vectors: Gene Therapy and Neuroscience Applications" (Kaplitt, M.G., and Loewy, A.D., eds.) Academic Press, San Diego, CA (1995).

These polynucleotide may include the incorporation of codons "preferred" for expression of the polynucleotides in selected nonmammalian hosts, e.g., prokaryotic or non-mammalian eukaryotic host cells.

Vectors may be used to introduce the polynucleotides of this invention into a host cell. Typically, these vectors also include transcription and translational initiation regulatory sequences operably linked to the polynucleotide that encodes an archaeal replication accessory factor. These vectors would facilitate the production of such a factor in a host cell.

In certain embodiments, to produce the replication accessory factor encoded by the polynucleotide, an appropriate promoter and compatible host cell may be chosen. Examples of compatible cells lines and expression vectors are well known in the art. Certain well known host cells are prokaryotes like *E. coli*, and *B. subtilis*. In a prokaryotic host cell, such as *E. coli*, a polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant polypeptide. Examples of eukaryotic host cells are yeast, fungi, plant, insect, amphibian, avian, or mammalian cells. See, e.g., "Gene Expression Technology" Methods in Enzymology, v. 185, D.V. Goeddel, ed. Academic Press Inc., San Diego, CA (1990).

In certain embodiments, one may employ a selectable marker in the host cell system or vector such that transformed cells are easily detectable. In certain embodiments, such markers are detectable after the cells have

been transformed. An example includes, but is not limited to, antibiotic resistance.

Those skilled in the art will be able to construct suitable expression systems in suitable host cells, especially in view of the many publications, including manuals, that discuss such information.

This invention provides a method for producing archaeal replication accessory factors by expressing a vector that comprises a polynucleotide that encodes a replication accessory factor in a suitable host cell and purifying the expressed product. Techniques using such host cells to express such polynucleotides are well known in the art. See, e.g., Sambrook et al. (1989).

This invention also provides recombinant protein produced by the above-described method.

The invention also provides isolated and purified archaeal replication accessory factors including, but not limited to, archaeal PCNA, archaeal RFC-P38, archaeal RFC-P55, archaeal RFA, archaeal CDC6, archaeal FEN-1, and archaeal helicases, e.g., helicase dna2, helicases 2 to 8, and archaeal MCM.

In certain embodiments, these accessory factors have part or all of the primary structural conformation and one or more of the biological properties of a replication accessory factor.

As used herein, the term "isolated and purified polypeptide" describes a polypeptide separate from at least one other protein that naturally accompanies the polypeptide. A polypeptide includes proteins and peptide sequences comprising at least two amino acids.

In addition to naturally-occurring allelic forms of the replication accessory factors, this invention also includes polypeptide analogs or fragments. One of skill in the art can readily design nucleic acid sequences that express such analogs or fragments of the replication accessory factors. For example, one may use well-known site-directed mutagenesis techniques to generate polynucleotides encoding such analogs or fragments. Those analogs and fragments will have one or more of the biological functions of the

naturally-occurring replication accessory factor, for example, without limitation, enhancing a nucleic acid polymerase reaction or complex formation.

Further, this invention provides a composition comprising at least one archaeal polypeptide for use in nucleic acid polymerase reactions. As used herein "nucleic acid polymerase reactions" includes, but is not limited to, PCR-based reactions that may include site-directed mutagenesis, amplification, labeling, and synthesis of nucleic acid of interest.

In certain embodiments of the invention, the composition further comprises at least one polymerase. Such a polymerase may include, but would not be limited to, Pfu polymerase, *P. furiosus* pol II polymerase, and/or Taq polymerase. Blends of different polymerases, such as blends of Taq and Pfu polymerases, are also within the scope of the invention.

In certain embodiments, the polymerase is a thermostable archaeal polymerase. The archaeal DNA polymerase may be obtained from archaea such as *Pyrococcus species GB-D, Pyrococcus species strain (kodakaraensis) KOD1, Pyrococcus woesii, Pyrococcus abysii, Pyrococcus horikoshii, Pyrodictium occultum, Archaeoglobus fulgidus, Sulfolobus solfatanicus* or *acidocaldarius, Sulfolobus acidocaldarium, Thermococcus litoralis, Thermococcus species 9 degrees North-7, Thermococcus species JDF-3, Thermococcus gorgonarius, Methanobacterium thermoautotrophicum. Methanococcus jannaschii, Methanococcus voltae, Thermoplasma acidophilum, Thermococcus fumicolans, Pyrobaculum islandicum, Aeropyrum, pernix, Desulfurococcus strain Tok,* and *Thermoccocus sp TY.* Related archaea from which the archaeal DNA polymerase may be obtained are also described in Archaea: A Laboratory Manual (Robb, F.T and Place, A.R., eds, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995).

According to certain embodiments, the polymerase is related to Pfu polymerase or *P. furiosus* pol II polymerase. Commercial enzymes that are

related to Pfu polymerase that are likely to function with the *P. furiosus* replication factors are; KOD (Toyoba), pfx (Life Technologies Inc.), Vent (New England Biolabs), Deep Vent (New England Biolabs), Pwo (Roche Molecular Biochemicals) and Tgo from *Thermoccocus gorgonarius* (GC RICH OCR System and High Fidelity PCR Master Mix, containing a polymerase blend with Taq polymerase, Roche Molecular Biochemicals). Additionally, archaea that contain genes that exhibit DNA sequence homology to *P. furiosus* pol II subunits are described in references (Makinjemi, M. et al. (1999) Trends in Biochem., Sci. 24:14-16; Ishino et al. (1998) J. Bacteriol., 180 2232-6). Those archaeal polymerases with sequence homology with *P. furosius* pol II are also likely to function with the replication accessory factors described herein.

In certain embodiments the archaeal factors are used with a thermostable eubacterial polymerase, or are used with a mixture of eubacterial and archaeal polymerases. Thermostable eubacterial polymerases may be related to the pol I, pol II, or pol III class of DNA polymerases. Thermostable pol I DNA polymerases have been described in *Thermus species (aquaticus, flavus, thermohilus HB-8, ruber, brokianus, caldophius GK14, Filiformis), Bacillus species (stearothermophilus, caldotenex YT-G, caldovelax YT-F), and Thermotoga maritima.* Commercial thermostable enzymes that are related to eubacterial pol I enzymes include Taq (Stratagene) Tth (Perkin Elmer), Hot Tub/Tfl (Amersham), Klen Taq (Clone Tech), Stoffel fragment (Perkin Elmer), UlTma (Perkin Elmer), DynaZyme (Finnzymes), Bst (New England Biolabs), and Bca (Panvera). Thermostable pol III DNA polymerases have been described in *Thermus aquaticus (Huang, et al. (1999) J. Mol. Evol. 48:756-69) and Thermus thermophilus (reference #13),* but could be obtained from other thermophilic eubacteria. Additional thermophilic eubacteria are described in the reference: "Thermophilic Bacteria," Kristjansson, J.K., CRC Press, Inc., Boca Raton, Florida, 1992.

In certain embodiments, to further enhance the nucleic acid polymerase reaction, the invention may also include an archaeal dUTPase (PEF) in the composition.

Unlike current methods of enhancing nucleic acid polymerase reactions, this invention also discloses a method of enhancing nucleic acid polymerase reactions comprising employing a composition comprising at least one archaeal replication accessory factor. Such method will enhance the synthesis, amplification, or mutagenesis of nucleic acids of interest.

According to certain embodiments, the archaeal polypeptides, including accessory factors, enhance any nucleic acid polymerization reaction. Such polymerization reactions include primer extension reactions, PCR, mutagenesis, isothermal amplification, DNA sequencing, and probe labeling. Such methods are well known in the art. Enhancement may be provided by stimulating nucleotide incorporation and reducing dissociation of the polymerase from the template. In addition, enhancement may be provided by reducing impediments in the nucleic acid templates, such as secondary structure and duplex DNA. Overcoming or improving such impediments through the addition of accessory factors like RFA, MCM, and helicase, can allow polymerization reactions to occur more accurately or efficiently, or allow the use of lower denaturation/extension temperatures or isothermal temperatures. RFA, MCM, and helicase should also facilitate synthesis of long targets, thus enhancing the nucleic acid polymerase reaction. According to certain embodiments, polymerase reactions can be enhanced by removal of secondary structure impediments by, for example, but without limitation, nucleases such as FEN-1 (*see, e.g.*, U.S. Patent Application 09/430,692, filed October 29, 1999). In yet other embodiments, RFA, MCM, and helicase may provide additional benefits in non-polymerizing applications which require single-stranded nucleic acids. For example, RFA may improve the specificity of protein/nucleic acid interactions.

According to certain embodiments, PCNA alone or with other accessory factors may enhance exonuclease reactions carried out by the 3' to 5' exonuclease activity of Pfu. Exonuclease reactions are used to prepare long single-stranded DNA templates. Enhancement may be provided by reducing dissociation of the polymerase from the template.

In addition to enhancing polymerization and exonuclease reactions, PCNA is expected to enhance repair processes that are mediated by archaeal polymerases, such as, but not limited to, Pfu or *P. furiosus* pol II, and that typically require additional repair proteins, such as FEN-1 and ligase.

According to certain embodiments, PCNA can also be used to enhance processes that are based upon the binding of nucleic acid sequences to complementary nucleic acid strands. For example, hybridization of labeled probes to complementary DNA or RNA strands is used in such methods as library screening, Southern blotting, Northern blotting, chip-based detection strategies, and Q-PCR detection strategies (e.g., molecular beacon hybridization probes). Such methods are well known in the art. *See, e.g.,* Sambrook et al. (1989); Current Protocols in Molecular Biology, (1993, including supplements through July, 2000). Increasing the stability of annealed probes by the addition of PCNA, alone or with at least one archaeal polypeptide, may enhance specificity of hybridization reactions by allowing more stringent hybridization conditions to be used, such as higher temperature and/or lower ionic strength. Increasing the stability of primer/template interactions may also allow one to carry out more efficient polymerization reactions using RNA polymerases, reverse transcriptases and other nucleic acid polymerizing enzymes.

According to certain embodiments compositions and methods comprising ligase, in combination with one or more additional archaeal polypeptides, including, without limitation, MCM, PCNA, RFC-P38, RFC-P55, RFA, CDC6, FEN-1, dUTPase, helicase 2, helicase 3, helicase 4, helicase5,

helicase 6, helicase 7, helicase 8, and helicase dna2, are provided for enhancing nucleic acid polymerase reactions.

The invention also provides kits designed to expedite performing the subject methods. Kits serve to expedite the performance of the methods of interest by assembling two or more components for carrying out the methods. Kits preferably contain components in pre-measured unit amounts to minimize the need for measurements by end-users. Kits preferably include instructions for performing one or more methods of the invention. Preferably, the kit components are optimized to operate in conjunction with one another.

In certain embodiments kits are provided for performing nucleic acid polymerase reactions that include at least one archaeal replication accessory factor, and possibly other proteins or compounds known to enhance such reactions. In certain embodiments, the kits may also include one or more polymerases. In certain embodiments, the kits are for synthesizing, amplifying, labeling, mutagenizing, or detecting nucleic acids of interest.

Certain embodiments of the invention are described in the following examples. However, these examples are offered solely for the purpose of illustrating the invention, and should not be interpreted as limiting the invention to these examples.

Experiments

Methods

1. Production of Accessory Factors from *Pyrococcus furiosus*

A. DNA Sequence Identification/PCR Primers.

The DNA sequences surrounding the DNA sequences of interest were examined for likely start and stop codons. The majority of DNA sequences of interest were identified in archaeal genome databases (*Pyrococcus horekoshi*, *Pyrococcus furious*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*), through similarity to eukaryotic genes encoding replication factors of interest (see reference No. 5). Also, the oligonucleotide sequence for PCNA was identified by N-terminal peptide sequencing of a

protein isolated from a native protein preparation (see below). Table 1 below lists PCR primers used to amplify the genes and to produce sequences that can be modified to produce cohesive ends with a cloning vector. The sequence corresponding to the vector sequence is underlined.

Table 1

Gene Name	Forward Primer	Reverse Primer
RFC P98/P38	GACGACGACAAGATGAGCGAAG AGATTAGAGAA (SEQ ID NO:)	GGAACAAGACCCGTTCACTTCTT CCCAATTAGGGT (SEQ ID NO:)
RFC P55	GACGACGACAAGATGCCAGAGC TTCCCTGGGTA (SEQ ID NO:)	GGAACAAGACCCGTTCACTTTTTA AGAAAGTCAAA (SEQ ID NO:)
PCNA	GACGACGACAAGATGCCATTCG AAATAGTCTTTG (SEQ ID NO:)	GGAACAAGACCCGTTCACTCCTC AACCCTTGGGGCTA (SEQ ID NO:)
RFC P98 Intein Deletion Primers*	ACTACAGCGGCTTTGG (SEQ ID NO:)	CTTTCCGACACCAGGG (SEQ ID NO:)
RFA	GACGACGACAAGATGATCATGA GTGCATTTACAAAAGAAGAAATA ATC (SEQ ID NO:)	GGAACAAGACCCGTTCACATCAC CCCCAATTCTTCCAATTCCC (SEQ ID NO:)
dna2 helicase	GACGACGACAAGATGAACATAAA GAGCTTCATAAACAGGCTT (SEQ ID NO:)	GGAACAAGACCCGTTCAAATGCT ATCCTTCGTTAGCACAACATA (SEQ ID NO:)
Helicase 2	GACGACGACAAGATGATTGAGG AGCTGTTCAAGGGATTAGAGAG TGAAAT (SEQ ID NO:)	GGAACAAGACCCGTTCATCTTTTT ACGGCAAATGCGAATTCTTCTCC CTT (SEQ ID NO:)
Helicase 3	GACGACGACAAGATGTTAATAGT TGTAAGACCAGGAAGAAAAAAGA	GGAACAAGACCCGTTCATCGTCT CTCACCCTTCAAAATTTTTCCTTC

	ATGA (SEQ ID NO:)	TTC (SEQ ID NO:)
Helicase 4	GACGACGACAAGATGCACATATT GATAAAAAAGGCAATAAAAGAGA GATT (SEQ ID NO:)	GGAACAAGACCCGTCTATTCCCA AAACTTTCTAGTTTGGATGTAGTG TTT (SEQ ID NO:)
Helicase 5	GACGACGACAAGATGTTATTAAG GAGAGACTTAATACAGCCTAGG ATAT (SEQ ID NO:)	GGAACAAGACCCGTCTACTCCTC ATCCTCTATATATGGGGCAGTTAT TA (SEQ ID NO:)
Helicase 6	GACGACGACAAGATGCTCATGA GGCCAGTGAGGCTAATGATAGC TGATG (SEQ ID NO:)	GGAACAAGACCCGTCTAGCTTAA CTTAAGTAAATGCCTATCTTTCTT CT (SEQ ID NO:)
Helicase 7	GACGACGACAAGATGATCGAAG GTTACGAAATTAAACTAGCTGTT GTAAC (SEQ ID NO:)	GGAACAAGACCCGTTCAAAAACC TTTCCCAGGTATGCGGGGGTCGC T (SEQ ID NO:)
Helicase 8	GACGACGACAAGATGAGGGTTG ATGAGCTGAGAGTTGATGAGAG GATA (SEQ ID NO:)	GGAACAAGACCCGTTCAAGATTT GAGAAAGTAATCAAGGGTACTTTT TCT (SEQ ID NO:)
Mcm	(5LICMCM) GACGACGACAAG*ATG*GACAGG GAGGAGATGATTGAGAGATTTGC AAAC (SEQ ID NO:) note: initial GTG changed to ATG (ital	(3LICMCM) GGAACAAGACCCGTTCAGACGGT TTTGTAGTAACCACTCTCTGGCAT (SEQ ID NO:)
mcm intein removal primers	(MCMINOUT1) AAGACCTGCTGCGGAACTACTTTT G (SEQ ID NO:)	(MCMINOUT2) ACTGCTGCAGCAGTTAGGGATGAG CG (SEQ ID NO:)
cdc6	(5LICCDC6) GACGACGACAAGATGAACGAAGG CATCAAATAAAGCTTGACGAG (SEQ ID NO:)	(3LICCDC6) GGAACAAGACCCGTTTAGATCAAC CTGCTCACTCTTAAGGGA (SEQ ID NO:)
FEN-1	GACGACGACAAGATGGGTGTCCC AATTGGTGAGATTATACCAAGAAA AG (SEQ ID NO:)	GGAACAAGACCCGTTTATCTCTT GAACCAACTTTCAAGGGTTGATT GTTTTCCACT (SEQ ID NO:)

B. PCR Amplification.

1) Procedure.

DNA sequences for PCNA, RFC P98/38, RFC P55, RFA, and helicases dna2 and helicases 2-8 were amplified with various PCR enzymes and polymerase blends using the primers in Table 1. The optimal amplification procedure is described below.

PCR Reaction Mixture:

10 μl 10x cloned Pfu buffer (Stratagene)

0.8 μl 100mM total dNTPs

3 μl mixed primers (100 ng/μl of each primer)

1.5 μl PfuTurbo DNA polymerase (2.5 U/μl) (Stratagene)

1 μl genomic or plasmid DNA (approximately 100 ng/μl)

83.7 μl H_20

2) Temperature Cycling.

Samples were amplified in a RoboCycler® temperature cycler (Stratagene). The extension time used was proportional to the amplification product size. Optimally, the extension time is 2 minutes per kilobase. The annealing temperature depended on the length and composition of the primers, which were usually designed with a T_m (melting temperature) between 50°C-60°C. A standad temperature cycling scheme is listed below:

95°C 1 minute 1 cycle

The following three steps are performed sequentially and are repeated for 30 cycles:

95°C for 1 minute; 50°C for 1 minute; 72°C for 2 minutes/kb of target.

C. Cloning of PCR Products.

1) PCR Product Purification.

Three to ten PCR reactions were generated for each DNA sequence. The PCR products were combined and purified with Stratagene's StrataPrep®

PCR purification kit according to its instructions. The purified products were examined on agarose gels (1% agarose/1XTBE) to verify product size and homogeneity. The gels were stained with ethidium bromide and visualized. If spurious bands were present, products of the correct size were isolated with Stratagene's StrataPrep® DNA gel extraction kit.

2) Insert Preparation (Ligation Independent Cloning (LIC) Method).

35 μl of purified PCR products were added to reactions containing:

5 μl 10x cloned Pfu buffer

1 μl 25 mM dATP

1 μl cloned Pfu polymerase (2.5 U/μl)

and the volume of each reaction was brought to 50 μl with 8 μl H_20. The samples were incubated for 20 minutes at 72°C in the RoboCycler® temperature cycler. This process allows the 3' to 5' exonuclease activity of the polymerase to remove bases at the 3' ends of the PCR products until a dA nucleotide is encountered. The presence of dATP in the reaction prevents further exonucleolytic degradation of the PCR product and the exposed 5' overhangs anneal precisely with the pCALnEK vector. This vector is available commercially from Stratagene and is used to produce annealing termini complimentary to the prepared insert.

3) Annealing.

The treated PCR products were allowed to come to room temperature before 40 μl of each prepared insert was added to separate tubes containing 40 ng of the LIC-ready pCALnEK vector. Samples were mixed and allowed to anneal for 16 hours at room temperature.

D. Transformation.

The annealed vector/inserts were transformed into competent cells, namely, Stratagene's Epicurian Coli® XL10-Gold® ultracompetent cells, and selected on LB- ampicillin plates. LB media is a commonly used reagent that

would be understood by those practiced in the arts. LB ampicillin plates are made by mixing:

10g NaC1
5g Yeast Extract
10g Tryptone
10g Agar.

Add H_20 to a final volume of 1 liter. Autoclave. Cool to 55°C. Add ampicillin to a concentration of 100 micrograms per ml. Mix well. Pour about 25 ml per plate.

Supercoiled DNA was isolated from the transformants using the instructions recommended in Stratagene's StrataPrep® Plasmid Miniprep Kit. The plasmids were used to transform BL21(DE3) CodonPlus® or BL2I (DE3) pLysS (Stratagene) cells. These cells were again selected on LB-ampicillin plates.

E. Preparation of Recombinant Protein.

1) Bacterial Expression of Recombinant Proteins.

The transformants were grown up in multiple liter batches from overnight cultures preferably in LB media supplemented with Turbo Amp™ antibiotic (Stratagene) at 100 μg/ml at 37°C with moderate aeration. When the cultures reached OD_{600} readings of 0.6 to 1.0, the cells were induced with 1 mM IPTG (Stratagene) and incubated in the same manner for 2 hours to overnight (16 hours). Induction causes the vector to produce recombinant protein with a calmodulin binding peptide (CBP) amino tag. The induced cells were collected by centrifugation and stored at -20°C.

Some helicase clones appeared to be unstable in BL21(DE3) cells. Supercoiled plasmids containing these helicases were transformed into BL21 CodonPlus® cells (Stratagene) and induced with bacteriophage Lambda CE6 (Stratagene) which contains the T7 RNA polymerase gene that provides significant production of protein in BL21 cells. Five to ten ml of $3x10^{10}$ plaque forming unit (pfu)/ml lambda CE6 stock (made according to provided

instructions in Lambda CE6 Induction Kit (Stratagene)) was used to induce 500 ml cultures for four hours at 37°C with moderate aeration.

2) Purification of Recombinant Proteins.

Frozen cells were resuspended to an approximate concentration of 0.25 g/ml in buffers identical or similar to calcium binding buffer: 50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 1 mM magnesium acetate and 2 mM $CaCl_2$. Cell suspensions were subjected to sonication three times with a Bronson Sonifier 250 at a duty cycle of 80% and an output level of 5 for 45 seconds. The suspensions were left on ice to cool between sonication events. The lysate was cleared by centrifugation at 26,890 g.

The cleared lysates were added to a milliliter of calmodulin agarose (CAM agarose), equilibrated in buffer. Recombinant protein was bound to the CAM agarose (Stratagene) via the CBP tag by incubation with gentle agitation at 4°C. After two hours, the reactions were centrifuged at 3000 x g for 5 minutes to collect the CAM agarose and recombinant protein. The lysate supernatant was removed and the CAM agarose was washed at least once by resuspending the resin in 50 ml of calcium binding buffer followed by collection of the CAM agarose by centrifugation at 3000 x g for 5 minutes, as described above. The CAM agarose was transferred to a disposable 15 ml column, packed, and then washed with at least 50 ml of the calcium binding buffer. Recombinant proteins were eluted from the column by using a buffer similar or identical to 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EGTA. Similar buffers may include 2mM EGTA and the salt requirement may vary from protein to protein. Certain proteins required elution at higher ionic strength using a buffer with 1M NaCl. Proteins were evaluated for size and purity using Tris-Glycine 4-20% acrylamide gradient gels (Novex) with an SDS loading dye (Novex). Gels were stained with silver or Sypro Orange (Molecular Probes).

F. Removal of Intein Sequences from Recombinant RFC P98 clone.

By alignment to eukaryotic RFC sequences, it was observed that the RFC P98 clone contained an intein sequence. In Figure 8, the intein sequences are marked in parentheses, and correspond to nucleotides 374 to 2028. Upon post-translational excision of the intein, the predicted size of the RFC subunit would decrease from 98 kDa to 38 kDa, and hence this RFC subunit is referred to as P38. To improve expression of recombinant P38 from the RFC P98 clone, the intein was removed by making 5' phosphate modified oligonucleotides that primed immediately upstream and downstream to the sequence coding for the intein termini (RFC P98 Intein Deletion Primers in Table 1). These primers were designed to have their 3' termini pointing away from the intein (inverse PCR). By using the RFC P98/pCALnEK plasmid as a template, all of the plasmid/insert sequence was amplified with the exception of the intein sequence. The PCR product was purified with StrataPrep PCR Purification Kit and ligated at room temperature for 16 hours prior to transformation, as described in section 1(D).

2. Protein Analysis Techniques.

A. Preparation of Antibodies.

Rabbit sera containing specific IgG was prepared by immunizing rabbits with the recombinant accessory factors. CBP-tagged fusion proteins were used to immunize 1-2 New Zealand white rabbits using the following immunization schedule: each rabbit was injected with 90-200 μg CBP-tagged fusion protein (as obtained from section 1(E)(2) above) in Complete Freund's Adjuvant (CFA), each rabbit was given a booster injection of 45-100 μg CBP-tagged fusion protein in incomplete Freund's adjuvant (IFA)18 days later; each rabbit was given a second booster injection of 45-100 μg CBP-tagged fusion protein in IFA 39 days later; and serum samples were obtained starting 45 days later and at various times thereafter.

B. SDS-PAGE.

Native and recombinant protein samples were analyzed on 4-20% acrylamide/2.6% bis-acrylamide Tris-Glycine gels (NOVEX), stained with

either silver stain or Sypro orange (Molecular Probes). Protein concentrations were determined relative to a bovine serum albumin (BSA) standard (Pierce), using Pierce's Coomassie Blue Protein assay reagent or by comparisons of relative staining intensities on SDS-PAGE gels.

C. Western Blot.

Protein samples were transferred from SDS-PAGE to nitrocellulose by electroblotting using standard techniques. The blots were blocked with 1% Blotto/TBS (instant milk in tris buffered saline) for 1 hour at room temperature, followed by incubation with immunized rabbit sera which had been diluted 1:500 or 1:1000 (1 hour). Blots were washed 3 times with TBS containing 0.01% Tween 20. The blots were then incubated for 0.5-1 hour with alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted 1:500 or 1:1000 in TBS-0.01% Tween 20. Finally, the blots were washed as before and then incubated in color development solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.3 mg/ml NBT, and 0.15 mg/ml BCIP) for approximately 1-10 minutes. The enzyme reaction was stopped and the membrane was washed five times with deionized water.

D. Amino acid sequence analysis.

Protein samples were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad). Blots were sent to Beckman Research Institute-City of Hope (Duarte, CA) for N-terminal amino acid sequence analysis.

3. Isolating DNA encoding Recombinant RFC from a Genomic Library (Alternative Method)

A *Pyrococcus furiosus* genomic library was plated on XL1-Blue MRF' *E. coli* (Stratagene) at a density of approximately 2000 plaques per plate. Duralose filters (nitrocellulose on nylon backing) were used to take replicate lifts from each plate. While the first filter was on the plate, orientation marks were made by stabbing a needle through the filter and into the plate. The

orientation marks were marked in pen on the back of the plate before the filter was removed. The filter lifts were treated as follows:

1.5-2.0 minutes	1.5 M NaCl, 0.5 M NaOH
2 minutes	0.5 M Tris (pH 8.0), 1.5 M NaCl
30 seconds	2xSSC, 0.2 M Tris (pH 7.5)

After treatment, the filters were partially dried until they were still damp, but no standing water was visible. The DNA was fixed onto the filters by UV crosslinking with the Stratalinker (Stratagene) set to the "Autolink" format according to the instructions.

The filters were prehybridized in 15 ml of:

5x SSC

40 mM $NaPO_4$ pH (6.5)

5x Denhardt's

5% Dextran Sulfate

50% Formamide

0.1 mg/ml Salmon sperm DNA (Boiled separately and added immediately prior to use)

Prehybridization was carried out at 42° C for approximately 2 hours.

Probe was generated from a 200 bp PCR product amplified from *Methanococcus jannaschii* genomic DNA using the following primers:

Oligo # 576: GATGAAAGAGGGATAGAT (SEQ ID NO:)

Oligo # 577: ATCTCCAGTTAGACAGCT (SEQ ID NO:)

These PCR primers were designed to anneal to regions flanking a 200 bp sequence of the *Methanococcus jannaschii* RFC gene that exhibits 52% amino acid identity to the RFC gene from human. (*See* Results Section 2 below).

The PCR product was purified from free primers, buffer, and nucleotides. Fifty ng of the product was labeled with ^{32}P-dATP using the Stratagene Prime-It II Random Primer Labeling kit. The probe was purified from free nucleotides before being boiled for five minutes and added to the

prehybridization reaction. The total probe ^{32}P-label was calculated to be 20 million counts per minute (cpm). Hybridization was allowed to continue overnight at 42° C before the hybridization solution was removed and the filters were washed four times with 0.1x SSC, 0.1% SDS at 60° C (very stringent conditions). The filters were exposed to X-ray film overnight and 20 primary isolates with strong signals on both replicate filters were picked.

Three primary isolates were diluted, plated, and screened again using the same method described above. Two filters produced positive lambda clones. Bluescript plasmid clones were excised from the lambda clones in SOLR cells (Stratagene) according to the manufacturer's instructions. The clones had inserts sizes of 8 kb and 10 kb. These plasmid clones were cut with Hind III, blotted, and probed with the original 200 bp *Methanococcus jannaschii* genomic RFC PCR amplification product discussed above. One positive truncated clone was isolated and sequenced from each end of the insert. The sequence showed two RFC sequences, specifically, the C-terminus of one sequence, and the N-terminus of another.

4. Production of Accessory Factors from Native Sources

A. *P. furiosus* extract.

Fermentation of *P. furiosus* DSM 3638 cells was carried out using the procedure described in Archaea: A Laboratory Manual, Robb, F.T. (editor-in-chief), Cold Spring Harbor Press, CSH, NY 1995. The cell paste was resuspended in lysis buffer (50 mM Tris-HCl (pH 8.2), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM beta-mercaptoethanol (-ME), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mg/ml aprotinin), lysed in a French press, and sonicated. The sonicate was centrifuged and the supernatant was collected for chromatography.

B. Column Chromatography.

The supernatant was chromatographed on a Q-Sepharose Fast Flow column (Pharmacia), equilibrated in 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, and 10 mM -ME. Flow-through fractions were collected, adjusted to pH 7.5,

and then loaded onto an SP Sepharose Big Bead column (Pharmacia), and equilibrated in buffer A (50mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.1% (v/v) Igepal CA-630, and 0.1% (v/v) Tween 20). The column was eluted with a 0-0.25 M KCl gradient/Buffer A. Fractions containing DNA polymerase activity were dialyzed, then applied to a Heparin Sepharose CL-6B column (Pharmacia), and equilibrated in Buffer B (same as buffer A, except pH 8.2). The column was eluted with a 0-0.3 M KCl gradient/Buffer B. Fractions exhibiting polymerase enhancing activity (nucleotide incorporation) (using the assay described below in Section 5(B)), or immunocrossreactivity (using the Western Blot described in Section 2(C) above) were identified. Native PCNA was further purified by excising the protein from SDS-PAGE gels.

C. Immunoaffinity chromatography.

Immunoaffinity columns were prepared using the ImmunoPure Plus kit (Pierce, Cat# 44893), following the manufacturer's method. Two milliliters (ml) of serum mixed with 2 mls of kit loading/binding buffer was used to prepare each column. Before using the column, the column and buffers were allowed to warm to room temperature.

1) Preparation of extract.

Five hundred milligrams frozen *P. furiosus* cells were used for each column. The cells were lysed in 2 ml lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, and 1 mM DTT), and sonicated twice for 2 minutes on ice. The cells were centrifuged at 26,890 g for 15 minutes and the supernatant was recovered. The lysate was mixed with an equal volume of binding buffer (10 mM Tris pH 8.0, 50 mM KCl, 0.1% Tween 80). This lysate was pre-cleared by incubation with 4 ml of protein A beads (Pierce cat # 20333) and equilibrated with binding buffer. The slurry was incubated at 4° C for 1 hour with agitation. The precleared extract was recovered by packing the beads into a disposable column and the flow-through was collected. The column was washed with 2 ml of binding buffer and the wash was collected and pooled

with the flow-through fraction. The final volume of pretreated lysate is about 6 ml. In some cases, the lysate was then run over a pre-bleed rabbit IgG control column to further clean up the lysate.

2) Immunoaffinity chromatography.

The column was equilibrated with 15 ml binding buffer (10 mM Tris HCl (pH 8.0), 50 mM KCl, 0.1% Tween 80), and then, the 6 mls of pre-cleared lysate was applied to the column. The column was washed with 10 ml binding buffer, followed by 10 ml wash buffer (10 mM Tris pH 8.0, 500 mM KCl, 0.1% Tween 80). Specific proteins were eluted with seven 1 ml washes of elution buffer (0.1 M glycine, pH 2.8). One ml fractions were collected in tubes. To each collection tube, was added 50 l 1M Tris pH 9.5 to raise the pH of the eluates as they were collected. After eluting the protein of interest, the column was washed with 4 ml of 1M Tris pH 8.0 and then 15 ml of binding buffer.

5. Biochemical Assays

A. Primer Extension Assay.

The *Pyrococcus furiosus* accessory proteins were tested for their ability to enhance a nucleic acid polymerase reaction comprising Pfu polymerase and primed single-stranded M13 DNA. One version of this assay provided for detecting extension products under non-denaturing conditions using ethidium bromide staining. For this assay, a reaction cocktail was made containing:

5 g/ml single-stranded M13 mp 18(+) strand DNA (Pharmacia cat# 27-1546-01)

275 ng/ml 40-mer primer (5' GGT TTT CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA GTG C 3') (SEQ ID NO:)

200 M each dNTP

1X cPfu buffer

water to 20 l.

Single stranded M13 DNA was mixed with primer, buffer, and water. The mix was heated to 95° C for 2 minutes and then cooled to room temperature. The rest of the reaction components were added. Each 20 l reaction contained 0.05 units of cloned Pfu polymerase and varying amounts of PCNA and RFC. For assessing RFC enhancement of polymerase reactions, assays contained 0.025 l of *P. furiosus* PCNA (about 1 ng), and varying amounts of native *P. furiosus* RFC. The reactions were incubated at 72° C for 15 minutes. Two l DNA loading dye (50% glycerol, 1xTBE, .05% bromphenol blue + .05% xylene cyanol) was added to each sample and 15 l of sample with dye was loaded in each well of a 1% agarose gel (Reliant, FMC cat# 54907) and electrophoresed. The gel was stained with ethidium bromide. The double-stranded M13 was seen as a brightly staining product that migrated higher than a 12 kb marker similar to the position where a double-stranded M13 DNA control migrates. In this assay, one looks for an increase in the size of products synthesized when PCNA, or PCNA combined with RFC, are added to Pfu. Ethidium bromide staining is proportional to the amount of double-stranded DNA produced from primed single-stranded M13 template.

A second version of this gel-based assay allows detection of radiolabeled extension products under denaturing conditions. The same template was used, except that the 40 bp primer was phosphorylated at the 5' end with [-^{32}P]ATP (>5000Ci/mmole) and T4 polynucleotide kinase. The labeled oligo was purified using a NucTrap probe purification column (Stratagene) and then annealed with single-stranded M13 at equimolar concentrations (100nM). The reaction cocktail comprised:

9.5g/ml single-stranded M13mp18 (+) strand DNA (Pharmacia)

52 ng/ml 40-mer primer, as above (SEQ ID NO:)

100M each dNTP

1x cloned Pfu DNA polymerase buffer

and water to 50l.

Single-stranded M13 DNA was mixed with primer, buffer and water. The mix was heated to 95°C for 2 minutes and then cooled to room temperature. The rest of the reaction components were added. Each reaction contained diluted cloned Pfu DNA polymerase, and varying amounts of PCNA, and RFC. Reactions were incubated at 72°C for varying times ranging from 1-30 minutes. The reactions were terminated by adding 3.3l of stop dye (95% formamide/20mM EDTA/ 0.05% bromophenol blue/0.05% xylene cyanol). The reaction mixtures were then subject to polyacrylamide gel electrophoresis using 6-8% denaturing gels. The gels were dried and exposed to autoradiographic film. The size of the full length extension product was determined by carrying out primer extension for 30 minutes with an excess of cloned Pfu DNA polymerase.

B. Stimulation of nucleotide incorporation.

The accessory factors were also tested for the capability of increasing dNTP incorporation by Pfu DNA polymerase or *P. furiosus* pol II DNA polymerase. This assay involves measuring dNTP incorporation into primed M13 DNA, by isolating and counting high-molecular-weight DNA bound to DE81 filter paper. A reaction cocktail was prepared as follows:

4 g/ml single-stranded M13mp18 (+) strand DNA (Pharmacia)

219 ng/ml 40-mer (see Section 5(A) above) (SEQ ID NO:)

1X cloned Pfu DNA polymerase buffer (Stratagene)

300 M each dGTP, dATP, dCTP

30 M dTTP

5 M ^{3}H-TTP (NEN NEG-221H)

To 10l of reaction cocktail was added either 0.025 units cloned Pfu Polymerase (Stratagene) or 0.05 units *P. furiosus* pol II. The *P. furiosus* pol II sequence was PCR amplified using the DNA sequences as described (Uemori et al., Genes to Cells 2:499-512 (1997)), and recombinant CBP-DP1/DP2 polymerase was cloned, expressed, and purified as described using the procedures outlined above in Section 1. To assay the temperature-dependence of PCNA enhancement (data in Figure 42), reactions were carried out for 10 minutes in the absence or presence of 100ng PCNA, using incubation temperatures ranging from 66-99°C.

The extension reactions were quenched on ice, and then 5l aliquots were spotted immediately onto DE81 filters (Whatman). Unincorporated [^{3}H]TTP was removed by 6 washes with 2xSCC (0.3M NaCl, 30mM sodium citrate, pH 7.0), followed by one wash with 100% ethanol. Incorporated radioactivity was measured by scintillation counting.

This assay can be modified to allow improved detection of PCNA by reducing dNTP incorporation to background levels through the addition of 200 mM KCL to the reaction mix. PCNA, alone or in combination with other accessory factors can be detected by restoration of Pfu's DNA polymerase activity.

The assay cocktail contains:

10 g/ml	single-stranded M13 mp 18 (+) strand DNA (Pharmacia cat# 27-1546-01)
100 ng/ml	40-mer primer (GGTTTTCCCAGTCACGACGTTGTA AAACGACGGCCAGTGC) (SEQ ID NO:)
1X	cPfu buffer
200 mM	KCl
30 M each	dATP, dCTP, dGTP
3 M	dTTP
5 M	^{3}H-dTTP (NEN cat# NET-221H) (100 Ci/mL)

100 U/ml cloned Pfu polymerase

Recombinant accessory factors or fractions derived from native *P. furiosus* were assayed for their ability to restore polymerase activity to the above cocktail. One l samples were added to 10 l of reaction cocktail, and reactions were incubated at 72° C for 30 minutes. The reaction mixtures were spotted onto DE81 filter papers, which were then washed and counted as described above.

C. ATPase assay.

One l of RFC or helicase preparations, obtained as described in Example 1E above, were incubated with 1 l of 4.5 M ATP and 1 Ci of gamma labeled ^{33}P-ATP (^{33}P--dATP) in 10mM Tris HCl (pH 8.3), 3.5 mM $MgCl_2$, and 75 mM KCl. The samples were incubated at 72° C for 20 minutes, then spotted on PEI cellulose F (EM Science). After drying, the PEI cellulose was placed in a shallow reservoir of 0.4 M NaH_2PO_4 pH 3.5 for thin layer chromatography (TLC). The liquid front was allowed to migrate 4-5 cm before being removed from the liquid and dried. The samples were exposed to X-ray film for one hour. The samples were determined to contain ATPase activity when the samples were observed to contain radioactivity migrating with the liquid front. The positive control (porcine ATPase) converts ^{33}P--dATP to dADP + ^{33}P- P. The ^{33}P- P product migrates with the liquid front under these TLC conditions, while the ^{33}P--dATP substrate remains near the origin. In some cases, product was quantified by excising the product spots from the PEI plate and then counting in a scintillation counter.

D. Gel shift assay.

A 38 base oligo (5' GGTTTTCCCAGTCACGACGTTGTAAAACGACGG CCAGT 3') (SEQ ID NO:) was incubated with RFA samples at 95ºC for 10 minutes, followed by 72ºC for 2 minutes, prior to loading on a 4-20% acrylamide gradient gel (Novex) in 1x TBE buffer. Bands were visualized by SYBER green staining (Molecular Probes) and UV illumination. DNA binding

activity is monitored by looking for a retardation in the migration of the oligo (higher band) in the presence of RFA. Single-strand DNA binding activity is verified by showing a shift in band position using a single-stranded oligo but no shift using a double-stranded DNA duplex. *See* Figure 18.

Pfu RFA was observed to contain a 4-cysteine-type zinc finger motif ($X_3CX_{2-4}CX_{12-15}CX_2C$; H is an acceptable substitute for C). While this motif seems unimportant for DNA binding in eukaryotic RFA, a recent publication reveals that oxidation of the cysteine residues causes significant diminishment of single-stranded DNA binding (Park et al., J. Biol. Chem. 274:29075-80 (1999)). Since the RFA-CBP, obtained in section 1.E.2 above, was purified in the absence of reducing agents we reconstituted the zinc finger motif under reducing conditions. One microliter of 100 mM DTT, 10 mM $ZnCl_2$was added to 10 L purified RFA-CBP protein in 50 mM Tris, pH 8.0, 125 mM NaCl. This mixture was heated at 74°C for 10 minutes to generate reconstituted RFA.

A gel shift assay was performed as follows. One or five L of the reconstituted RFA was mixed with nine L of a solution containing 5.5ng/µl of the single-stranded DNA oligomer GAGAGAATTCATAATGATAAGGAGGAAAAAATTATGATCCTGGACGATGACTACATCACC (SEQ ID NO:) in 10 mM Tris, pH 8.0 with or without 0.1 mM EDTA. The samples were incubated at room temperature for 5 minutes then mixed with 3µl of 6x loading dye and loaded on a 4-20% gradient acrylamide gel. When the bromophenol blue dye had migrated to 2/3rds of the gel length, the gel was stained in SYBR green dye (Molecular Probes, Eugene, OR) and examined under UV light.

As seen in Figure 54, the reconstituted RFA caused the electrophoretic mobility of the single-stranded oligonucleotide to decrease, indicating that the reconstituted RFA bound to the oligomer. Lane 1 shows the electrophoretic migration of the oligomer in water (negative control). Lane 2 shows the

migration of the oligomer in the presence of 1 g *E. coli* SSB (positive control). Lanes 3 and 4 show the migration rate of the oligomer in the presence of 1 g (lane 3) or 5 g (lane 4) Pfu RFA-CBP protein. Thus, under these conditions, the reconstituted Pfu RFA-CBP was approximately as effective in binding to the oligomer's migration as the control *E. coli* SSB protein (note that *E. coli* SSB appears to quench some of the SYBR green signal intensity, cp. lanes 2 and 3).

E. Helicase assay.

Radioactively labeled oligonucleotides with a 3' overhang or a 5' overhang were annealed to M13mp18 DNA (Pharmacia). The reactions were incubated with 0.5 l of putative *P. furiosus* helicases in 50 mM Tris HCl, pH 8.5, 25mM KCl, and 5 mM ATP for 30 minutes at 55ºC. The positive control was generated by thermally melting the annealed oligo prior to loading. The samples were run on 4-20% gradient acrylamide gels in 1x TBE. The gels were dried and exposed to X-ray film. Samples with helicase activity will displace the annealed radiolabeled oligo from single-stranded M13 DNA. The helicase-displaced oligo will migrate in a gel with the same mobility as oligos melted off M13 DNA with heat (free oligo). In samples lacking helicase activity, the oligo will still be bound to M13 and will migrate at a different position, similar to the "template only" controls.

6. PCR Reactions.

PCR reactions were carried out under standard conditions. In general, amplification reactions (50 l) contained 200-500 M each dNTP, 1x PCR buffer, 50-250 ng of human genomic DNA template (or 100-200 ng Stratagene's Big Blue transgenic mouse genomic DNA for the 0.5 kb target), 100 ng of each primer, and 2.5-5U of TaqPlus® Long DNA polymerase blend, PfuTurbo DNA polymerase, or Taq2000 DNA (Stratagene) polymerase. TaqPlus® Long PCRs were carried out in 1x buffer including: 50 mM Tricine pH 9.0, 8 mM ammonium sulfate, 0.1% Tween-20, 2.3 mM $MgCl_2$, and 75 g/ml BSA. PCRs using PfuTurbo or Taq2000 DNA polymerase were carried

out with the PCR buffers provided with the enzymes (Stratagene). Amplifications of targets ≥17kb with PfuTurbo DNA polymerase employed 1.5x reaction buffer and 500μM each dNTP (Borns, M. et al., Strategies Newsletter 13:27-30, 2000). The sequences of the PCR primers used to amplify the 17kb β-globin target are shown below. Reactions were cycled in 200 l thin-walled tubes using any of the following temperature cyclers: Stratagene RoboCycler® 96 temperature cycler fitted with a hot top assembly, Perkin Elmer GeneAmp PCR System 9600, or MJ Research PTC-200 Peltier thermocycler.

The PCR primer sequences were:

17 kb -globin

forward primer: 5'- CACAAGGGCTACTGGTTGCCGATT (SEQ ID NO:)

reverse primer: 5'- CAGGGCATTGACAGCAGTCTTCTCCTCAGG (SEQ ID NO:)

23 kb -globin

Forward primer: 5'-CACAAGGGCTACTGGTTGCCGATT (SEQ ID NO:)

Reverse primer: 5'-AGCTTCCCAACGTGATCGCCT (SEQ ID NO:)

30 kb -globin

Forward primer: 5'-CTCAGATATGGCCAAAGATCTATACACACC (SEQ ID NO:)

Reverse primer: 5'-AGCTTCCCAACGTGATCGCCTT (SEQ ID NO:)

2.1 kb Alpha 1 Anti-Trypsin

Forward primer: 5'-GAGGAGAGCAGGAAAGGTGGAAC (SEQ ID NO:)

Reverse primer: 5'-GAAAATAGGAGCTCAGCTGCAG (SEQ ID NO:)

5.2 kb Alpha 1 Anti-Trypsin

Forward primer: 5'-GAGGAGAGCAGGAAAGGTGGAAC (SEQ ID NO:)

Reverse primer: 5'-GCTGGGAGAAGACTTCACTGG (SEQ ID NO:)

0.5kb /lacI (transgenic mouse genomic DNA)

lambda primer: 5'-GACAGTCACTCCGGCCCG (SEQ ID NO:)

lacZ primer: 5'-CGACGACTCGTGGAGCCC (SEQ ID NO:)

The following temperature cycling conditions were used for the 23 and 30kb -globin targets: 92°C for 2 min. (1 cycle); 92°C for 10 sec., 65°C for 30 sec, 68°C for 25 min. (10 cycles); 92°C for 10 sec, 65°C for 30 sec, 68°C for 25 min. (with a increase of 10 sec. added progressively to the extension time with each cycle)(20 cycles). The 2.1 and 5.2 kb targets were amplified as follows: 95°C for 1 min. (1 cycle); 95°C for 1 min., 58°C for 1 min., 72°C for 2 min. (for 2 kb target) or 5 min. (for the 5.2 kb target) (30 cycles); 72°C for 10 min. (1 cycle). The 0.5 kb target was amplified as follows: 94°C for 1 min. (1 cycle); 94°C for 1 min., 54°C for 2 min., 72°C for 1.5 min. (30 cycles); 72°C for 10 min. (1 cycle).

Results

1. PCNA.

P. furiosus PCNA was first identified in column fractions produced during fractionating native *P. furiosus* extracts. PCNA was co-purified with Pfu DNA polymerase during the Q and SP column procedures discussed above. Peak PCNA activity could be resolved from peak DNA polymerase activity using the heparin sepharose column, but all PCNA-containing fractions were contaminated with DNA polymerase activity.

To isolate native PCNA, fractions that could restore DNA polymerase activity to salt-inactivated Pfu DNA polymerase were studied. Such "restoration" activity was detected in column fractions eluting off the Heparin sepharose (Figure 1). An active column fraction was then subject to SDS-PAGE and gel slices were excised and extracted to remove proteins. DNA polymerase activity was found in a gel slice recovered from a position in the gel corresponding to the migration of proteins between 64-98 kDa. In contrast, PCNA activity was recovered from a gel slice that was located at a position lying between the 30 and 36 kDa protein markers (Figure 2). A protein band, migrating at 35 kDa, was visible on SDS-PAGE gels. This protein was transferred to a PVDF membrane (Bio Rad, Hercules, CA) and

sent for amino-terminal sequencing. The N-terminal sequence of the 35 kDa protein was: PFEIVFEGAKEFAQLIDTASKL(H/I)DEAAFKVTEDG--MR (SEQ ID NOs:) (where (H/I) means either amino acid could be present, and - means that any amino acid could be present). A BLAST search of DNA sequence databases identified the 35 kDa protein as exhibiting significant homology to known eukaryotic PCNA sequences.

The sequence encoding *P. furiosus* PCNA was cloned in the pCALnEK vector using the PCNA PCR primers shown in Table 1. The primers were designed using the DNA sequence for PCNA identified in the *Pyrococcus horekoshi* genome sequence database. *Pyrococcus horekoshi* is closely related to *Pyrococcus furiosus*. The translated N-terminus of the putative *Pyrococcus horekoshi* PCNA matches the chemically determined N-terminal sequence of native *Pyrococcus furiosus* PCNA. The DNA sequence of the pCALnEK clone encoding *Pyrococcus furiosus* PCNA is shown in Figure 3, and its predicted amino acid sequence is shown in Figure 4. The predicted molecular weight of *P. furiosus* PCNA is 28 kDa although the apparent molecular weights of EK-digested recombinant PCNA and native PCNA are 38 and 35 kDa, respectively.

In addition to stimulating nucleotide incorporation by salt-inactivated Pfu DNA polymerase, both native and recombinant *Pyrococcus furiosus* PCNA preparations were shown to significantly increase the processivity of Pfu. When PCNA is added to primer extension reactions comprising a 5' radiolabelled primer annealed to single stranded M13 template, the majority of the products generated at early time points are full-length and fewer short truncated products accumulated (Figure 5). These results indicate that PCNA has significantly enhanced this nucleic acid polymerase reaction by increasing the processivity of Pfu polymerase (number of bases added per polymerase/DNA binding event), and increasing the overall rate of nucleotide incorporation (nucleotides incorporated per unit time).

The effects of PCNA on *P. furiosus* pol II DNA polymerase were also tested. PCNA was shown to stimulate dNTP incorporation by both Pfu polymerase and *P. furiosus* pol II DNA polymerases. Interestingly, the addition of PCNA altered the optimal reaction temperature for both DNA polymerases. Because DNA duplexes are unstable at elevated temperatures, performing nucleic acid polymerase reactions at temperatures approaching the optimal growth temperatures of hyperthermophilic archaea (>100°C) has been difficult. For the M13/40-mer duplex shown in Figure 42, reaction temperatures above 75°C produce template instability, consistent with the drop in activity for both polymerases between 72 and 80°C. However, in the presence of PCNA, the primer/M13 duplex appears to be stabilized at temperatures > 72°C, leading to even higher primer extension activity by both Pfu (pol I) and *P. furiosus* pol II DNA polymerases. Thus, the M13/oligo duplex remains annealed at temperatures greater than about 80°C.

These data indicate that the addition of PCNA can have other benefits besides enhancing the polymerization rate and processivity of Pfu (pol I) and *P. furiosus* pol II DNA polymerases. The use of PCNA should allow the use of these hyperthermophilic enzymes at higher temperatures than has been achieved to date, thereby enhancing certain nucleic acid polymerase reactions. PCR amplification, DNA sequencing, and isothermal amplification reactions employ extension temperatures of $\leq$ 72°C to ensure stability of the primer/template duplex. However, this temperature is well below the expected temperature optimum of DNA polymerases from hyperthermophilic archaea like *P. furiosus*. It may be possible to use elevated extension temperatures during these polymerization reactions (e.g., >80°C), which would have the benefits of increasing polymerase activity (by operating closer to optimum reaction temperature) and reducing interference from secondary structure in DNA templates.

In addition, the apparent stabilization of primer/M13 DNA duplexes by PCNA may have utility in improving applications that require high stability of

nucleic acid duplexes. For example, PCNA may enhance the specificity of probe hybridization reactions by allowing the use of more stringent annealing temperatures or reaction conditions (lower ionic strength).

The effect of adding PCNA without other accessory factors to PCR amplification reactions has been tested. In the absence of other accessory factors, relatively high concentrations of PCNA (100 ng-1 ug) can inhibit product synthesis by Pfu DNA polymerase. Lower concentrations of PCNA are tolerated in PCR amplification reactions (<100 ng). PCNA is functional and beneficial to PCR amplification reactions (Figures 6 and 7). PCNA can dramatically increase the yield of products amplified with DNA polymerase blends including *Taq, Pfu,* and *P. furiosus* dUTPase (PEF). In the blends that have been tested, Taq is present at 2.5-5U and Pfu is present at 0.156-0.3125 U. The dUTPase may be in the form of native PEF or recombinant dUTPase (P45) (See WO 98/42860; U.S. Patent Application Serial No. 08/957,709) and present at 1-10 ng per reaction. PCNA enhances the processivity of the minor proofreading component in the DNA polymerase blend, while dUTPase is preventing dUTP incorporation (and subsequent Pfu inhibition). Thus nucleic acid polymerase reactions comprising Pfu polymerase are enhanced. The dUTPase activity is discussed in International Patent Application WO98/42860 and U.S. Patent Application Serial No. 08/957,709, which are expressly incorporated by reference in their entirety for any purpose. Therefore, optimal concentrations of PCNA should enhance nucleic acid polymerase reactions comprising archaeal DNA polymerases (such as Pfu, *P. furiosus* pol II), either alone, in combination, or blended with other non-proofreading DNA polymerases of eubacterial or archaeal origin, such as blends of Pfu and Taq (*See* U.S. Patent No. 5,556,772, U.S. Patent Application Serial No. 08/529,767, filed September 18, 1995, and U.S. Patent Application Serial No. 09/414,295, filed October 6, 1999. In addition, PCNA activity can be improved by the further addition of other accessory factors including *P. furiosus* RFC, RFA, MCM, CDC6, FEN-1, and helicase.

2. RFC.

Before this invention, the inventors were not aware of the availability of an archaeal genome sequence other than the sequence of *Methanococcus jannaschii.* The genome sequence of *Methanococcus jannaschii* contained open reading frames (ORFs), which exhibited significant DNA sequence homology to DNA replication proteins from eukaryotes, including one Family B DNA polymerase, two RFC subunits, and PCNA (Bult et al., 1996 (Reference No. 6)). In eukaryotes, the RFC complex is composed of five distinct subunits (one large subunit and 4 small subunits that are associated with ATPase activity) and is stimulated by PCNA. However, only two genes were identified in *Methanococcus jannaschii* as exhibiting homology to RFC subunits: one sequence was identified as a putative homolog of the large RFC subunit and a second sequence was identified as a putative homolog of one of the small subunits. Initially, PCR primers were based upon the DNA sequences of the putative *Methanococcus jannaschii rfc* genes. However, these primers did not amplify a PCR product from *P. furiosus* genomic DNA, presumably because of the divergence in DNA sequence between *Methanococcus jannaschii* and *Pyrococcus furiosus.*

The inventors used an alternative approach to clone *P. furiosus* RFC subunits. Amino acid sequence alignments between *Methanococcus jannaschii* and human RFC identified a 67-amino acid region with 52% identity. A portion of RFC was likely to be highly conserved among archaea, since it was relatively conserved between more distantly related organisms, i.e., humans and archaea.

A 200 bp sequence encompassing the region encoding the homologous 67-amino acid region was amplified from *Methanococcus jannaschii* genomic DNA using the following primers: 5' GATGAAAGAGGGATAGAT (SEQ ID NO:) and 5' ATCTCCAGTTAGACAGCT (SEQ ID NO:). The *Methanococcus jannaschii* sequence was used to probe a *P. furiosus* genomic DNA library.

One positive genomic clone was recovered which contained the sequences encoding both the large and small subunits in tandem. The DNA sequence of the genomic clone is shown is Figure 8 and the predicted amino acid sequence is shown in Figure 9. The genomic sequences of P38 and P55 are bracketed. The nucleotide sequence of P38 is nucleotides 197 to 2835 (the intein sequence is nucleotide 374 to 2028). The nucleotide sequence of P55 is nucleotides 2839 to 4281. Examination of the DNA sequence encoding the small *P. furiosus* RFC subunit (P98) revealed the presence of an intein. An intein had also been identified in the gene encoding the putative *Methanococcus jannaschii* small RFC subunit (Bult *et al*, 1996).

Expression constructs were prepared by subcloning the sequences encoding the large and small subunits into the pCALnEK vector. To facilitate expression, the intein was removed from the small RFC subunit clone by amplification with primers designed to anneal to the 5' and 3' regions flanking the intein sequence and to prime in a direction opposite to the intein. The predicted amino acid sequences of the large RFC subunit and the small "intein-less" RFC subunit are shown in Figures 10 and 11.

Antibodies were raised in rabbits against the P55 and P38 subunits. The native RFC complex was purified from *P. furiosus* extracts by immunoaffinity chromatography using either immobilized anti-P55 or anti-P38 IgG. Western blot analysis of immunoaffinity-purified RFC complex shows the presence of both subunits regardless of the capture antibody (Figure 12), indicating that P38 and P55 form a complex in *P. furiosus* as do the large and small RFC subunits in eukaryotes. The protein composition of one native RFC preparation is shown in Figure 13. In addition to P55 and P38, other unidentified protein bands are present.

The ATPase activity of the RFC preparations was tested as shown in Figure 14. The RFC subunits were determined to possess ATPase activity. That is, they convert ATP to ADP and phosphate. The RFC complex in eukaryotes is known to load the PCNA "sliding clamp" onto DNA in a process

that typically requires the conversion of ATP to ADP and phosphate. Recombinant P55 and P38 exhibited ATPase activity when assayed separately. A mixture of P55 and P38 subunits was also found to exhibit ATPase activity that increased in the presence of PCNA, but not in the presence of primed M13 DNA. In contrast, native RFC purified by immunoaffinity chromatography exhibited ATPase activity which was further stimulated by both PCNA and DNA. As the eukaryotic RFC complex is stimulated by both PCNA and DNA, it appears that the native RFC preparation is fully functional, while the mixture of recombinant P55 and P38 may be only partially active.

This conclusion was supported by primer extension studies in which an immunopurified native RFC preparation from *P. furiosus* was shown to enhance the yield of full-length products synthesized in a reaction comprising Pfu DNA polymerase and PCNA (Figure 15). In contrast, a mixture of recombinant P55 and P38 with similar ATPase activity showed less enhancement of primer extension by Pfu + PCNA. It is presently unknown whether the difference in activity between native and recombinant RFC is due to differences in the P55:P38 ratios or protein modification, or to the absence of additional proteins present in the native RFC preparations. One skilled in the art could readily determine a solution by attempting different ratios of P55 to P38 or different reaction conditions, or by adding additional protein factors such as the ones present in a native RFC preparation. Since the two RFC genes are adjacent in the Pfu genome, it may be necessary to co-express the cloned proteins to promote assembly of a fully functional RFC complex. Additionally, the presence of the CBP affinity tag could cause abnormal protein folding which would not spontaneously revert to the native structure when the tag is removed. The skilled artisan would appreciate that neither of these approaches would require undue experimentation and/or methodology other than as provided herein.

3. RFA.

The large subunit of eukaryotic RFA was used to search the archaeal protein databases with PSI-BLAST. Hits to archaeal sequences were examined. The inventors aligned corresponding sequences to identify the putative start and stop codons of the RFA sequence in the incomplete *P. furiosus* genome sequence. The *P. furiosus rfa* sequence was PCR amplified and cloned into the pCALnEK vector. The DNA sequence and predicted amino acid sequences are shown in Figures 16 and 17. The apparent molecular weight of the expressed fully denatured protein was consistent with the size expected from the predicted amino acid sequence (41 kDa).

To assess function, *P. furiosus* RFA was tested for single-stranded DNA binding activity in a gel shift assay (Figure 18). When RFA was incubated with a 38 base oligonucleotide, the migration of a percentage of the DNA was reduced, indicating that RFA does exhibit single-stranded DNA binding activity. In comparison, *E. coli* SSB was found to completely retard the oligo. The weaker single stranded DNA binding activity exhibited by *P. furiosus* RFA may be explained by use of insufficient protein, the presence of the CBP tag, or the use of suboptimal reaction conditions. The solutions used in the purification of CBP-RFA contained no reducing agents which may have caused reversible protein denaturation. Oxidation of the zinc-finger domain of eukaryotic RFA has been shown to reduce single-strand DNA binding capacity. (Park et al.) As shown in figure 54, when the Pfu CBP-RFA was reconstituted with reducing agent and $ZnCl_2$, the gel shift is similar to that of E. coli SSB.

The degree of migration of the oligo is related to the mass of the protein-DNA complex and the formation of protein multimers. *E. coli* SSB is known to form tetramers, but it is presently unknown whether *P. furiosus* RFA also forms multimers.

The addition of *P. furiosus* RFA to amplifications carried out with Pfu and Taq DNA polymerases was shown to increase amplification specificity

(Figures 19 and 21) and PCR product yield (Figures 20 and 21). The conditions were as described in Section 6 above. In Figure 21, *P. furiosus* RFA produced effects which were similar to those generated by *E. coli* single-stranded DNA binding protein (SSB; Stratagene's Perfect Match), including increased yield and amplification specificity and retardation of DNA migration at excess concentrations (5 l). No evaluation of the relative performances of *P. furiosus* RFA and *E. coli* single-stranded DNA binding protein in PCR has been made; however, the increased thermostability of RFA should provide an additional benefit in temperature cycling.

4. Helicase.

Cells contain multiple helicases with specialized roles in a number of processes including replication, DNA repair, recombination, transcription, and translation. Known helicases have been classified into five families based upon sequence homology. Mechanistically, there are 2 classes of helicases depending upon whether unwinding requires a 3 ' overhang (3'-5' polarity) or a 5' overhang (5'-3' polarity), which is characteristic of helicases functioning in DNA replication. Archaeal replicative helicases were identified by identifying those ORFs in archaeal genomes that exhibited homology to known eukaryotic helicases, regardless of specific metabolic role. No putative helicase sequences were excluded because helicase function between archaea and eukaryotes may be different. Moreover, the eukaryotic replicative helicase has not been conclusively identified. Using eukaryotic helicases, a PSI-BLAST search in the archaeal protein databases was conducted.

Eight putative helicases meeting the criteria were selected for analysis. The incomplete *P. furiosus* genome sequence was examined to identify the putative start and stop codons of these sequences and to design PCR primers for cloning. The DNA sequences are shown in Figures 22-28, and 40, respectively; the predicted amino acid sequences are shown in Figures 29-35, and 41, respectively. The apparent molecular weights of the

expressed proteins were consistent with the sizes expected from the translated DNA sequence (see figure description of Figures 29-35). Future corrections in the incomplete *P. furiosus* genome sequence may define alternative start and stop sites.

Helicases act to displace the complimentary strand of DNA or RNA to uncover template for DNA polymerases, RNA polymerases, accessory factors, and repair factors. Helicases melt the complimentary strand in a process coupled to hydrolysis of ribo- or deoxyribonucleotides. Most helicases displace either a 5' overhang or a 3' overhang, but some helicases displace both templates or utilize different templates under different reaction conditions. Typically, a helicase will utilize one or more nucleotide triphosphates preferentially. To assess the function of the identified eight helicases, recombinant helicases were tested for ATPase activity. The ribonucleotide ATP was used, although other ribo- or deoxyribonucleotides may serve as the preferred substrate. The resulting recombinant proteins were incubated with ATP, and phosphate was detected after separation by TLC. The results in Figures 36 and 37 demonstrate that all eight recombinant helicases exhibit ATPase activity.

Eight recombinant helicases were tested for helicase activity. The templates used included labeled oligonucleotides annealed to single-stranded M13mp18 DNA. The oligos had either 5' or 3' non-complementary ends. As shown in Figure 38, helicase 2 was able to displace oligos from both templates. This helicase also melted a template which had non-complementary 5' and 3' ends (data not shown). Such a forked template mimics the "bubble" formed by the replication fork. In addition, helicase 7 displaced the oligo with a free 3' end (Figure 38). The lack of detectable oligo displacement does not necessarily mean that the rest of the enzymes are not helicases. For example, helicase activity may not be detected due to the use of suboptimal buffers or reaction conditions, the presence of the N-terminal CBP tag, or the use of insufficient amounts of recombinant protein.

Preliminary experiments demonstrated that the addition of diluted preparations of helicase 2 or helicase dna2 to PCR reactions comprising Pfu DNA polymerase can lead to increased PCR product yield.

5. MCM and CDC6

Nucleotide sequences corresponding to the *P. furiosus* MCM coding region are shown in Figure 45 (intein present) and Figure 55 (intein sequences removed). The predicted amino acid sequences are shown in Figures 46 (intein present) and 47 (intein removed). The apparent molecular weight of recombinant *P. furiosus* MCM as determined by SDS-PAGE was 82kD, consistent with the expected migration for MCM-CBP (calculated molecular weight: 76.8kD *P. furiosus* MCM + 4kD CBP tag). The MCM interin was removed as described in Example 1F, above, except that the *mcm* intein removal primers, shown in Table 1 were used.

The nucleotide and predicted amino acid sequences of *P. furiosus* CDC6 are shown in Figures 48 and 49, respectively. The apparent molecular weight of recombinant *P. furiosus* CDC6 as determined by SDS-PAGE was 53kD, consistent with the expected migration for CBP-CDC6 (calculated molecular weight: 48.2kD *P. furiosus* CDC6 + 4kD CBP tag). Other higher molecular weight bands were also evident by SDS-PAGE, suggesting CDC6 proteins also produce multimers.

The *P. furiosus* MCM was found to increase yields of PCR products amplified with TaqPlus Long DNA polymerase, a Taq plus Pfu DNA polymerase blend (Stratagene), according to Method 6 (PCR Reactions), above . As shown in Figure 53, MCM alone significantly improved yields of a 23kb genomic target over a $\geq$10-fold range of protein concentrations (Figure 53, left panel). CDC6 alone, in contrast, increased product yields only slightly. However, the combination of CDC6 and MCM produced significantly higher product yields than could be achieved by either factor alone (Figure 53, right panel). These observations were unexpected in light of the reported inhibitory effects of *Mth* CDC6/ORC homologs on the helicase activity of *Mth*

MCM (Kelman et al., Proc. Natl. Acad. Sci. 96:14783-88 (1999)). It is possible that *P. furiosus* MCM and/or CDC6 homologs improve amplification reactions through mechanisms other than helicase activity and helicase loading, respectively. For example, the DNA binding activities of MCM and/or CDC6 proteins may contribute to improved DNA synthesis by stabilizing ssDNA templates and thereby reducing structural impediments that may impede translocation of Pfu DNA polymerase.

Additional studies have shown that MCM and CDC6 also enhance yields of products amplified with PfuTurbo DNA polymerase (data not shown). Yield improvements appear to be most dramatic in amplifications of longer targets (>5kb). The experimental conditions for PfuTurbo amplification are as described above in Method 6 (PCR Reactions). While little enhancement of PfuTurbo amplification of 2.1 or 5.2 kb targets is apparent when MCM and CDC6 are added, there is marked enhancement when longer targets, such as the 17 kb -globin target, are amplified in the presence of Pfu MCM alone or in combination with CDC6.

6. FEN-1

A. PCR.

Recombinant *P. furiosus* FEN-1 was added to PCR amplification reactions carried out as described above, except that amplifications of targets ≥17kb with PfuTurbo DNA polymerase employed 1.5x reaction buffer and 500μM each dNTP (Borns, M. et al., Strategies Newsletter 13:27-30, 2000). The sequences of the PCR primers used to amplify the 17kb β-globin target were as follows:

forward primer: 5'- CACAAGGGCTACTGGTTGCCGATT (SEQ ID NO:)
reverse primer: 5'- CAGGGCATTGACAGCAGTCTTCTCCTCAGG(SEQ ID NO:).

B. Enhancement of PCR.

The benefits of FEN-1 are particularly evident in amplifications of longer genomic targets. As shown in Figure 50, a 23kb target was amplified from genomic DNA using TaqPlus Long DNA polymerase, as described in Method 6 (PCR Reactions) above. Product yields increased with FEN-1 alone, and were further increased when PCNA and FEN-1 were added in combination. The final concentrations of PCNA and FEN-1 present in each reaction mixture are indicated in Figure 50.

Stimulation is achieved with relatively low concentrations of FEN-1 and PCNA, while relatively high concentrations can lead to inhibition. For example, in Figure 50 amplifications were completely inhibited when 4.8nM PCNA was employed, and reduced enhancement was noted when >1.2nM FEN-1 was used.

FEN-1 stimulation of PCNA activity was also observed using PfuTurbo DNA polymerase and a 17kb genomic -globin DNA target (Figure 51) using the method described in Method 6 (PCR Reactions) above. A 17kb target was amplified from human genomic DNA with PfuTurbo DNA polymerase in the absence (0) of PCNA and FEN-1, the presence of PCNA alone (11 fg/ l PCNA (131 pM PCNA)), or the presence of PCNA and FEN-1 (11 fg/ l PCNA (131 pM PCNA) and 30 fg/ l FEN-1 (774 pM FEN-1). Amplifications were carried out in the absence (0) or presence of varying concentrations of a thermostable topoisomerase (ThermoFidelase; Chemicon). For the topoisomerase, 1ul of the dilution indicated at the top of the gel was added per 50ul PCR reaction.

The topoisomerase was inhibitory, but the stimulatory effect of PCNA and FEN-1 allowed partial reversion of inhibition. PCNA alone increased

yield, and further increases were noted with the addition of FEN-1, as shown in Figure 51.

A 17kb target was also amplified from human genomic DNA with PfuTurbo DNA polymerase in the absence (0) or presence of PCNA and varying concentrations of FEN-1 (Figure 52). FEN-1 provides a slight increase in yield, and the addition of PCNA further increases product yield. In this example, FEN-1 concentrations above 0.1nM were less effective than concentrations of 0.01-0.1nM.

All documents mentioned in this application, including but not limited to, articles, books, reviews, patents and patent applications, are hereby incorporated by reference into this specification in their entirety for any purpose.

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Claims

1. A composition for enhancing nucleic acid polymerase reactions comprising an archaeal MCM and at least one archaeal polypeptide selected from the group consisting of: PCNA, RFC-P38, RFC-P55, RFA, CDC6, FEN-1, dUTPase, ligase, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, and helicase 8.

2. The composition of claim 1, wherein the archaeal MCM is from or homologous to MCM found in *Pyrococcus furiosus*.

3. The composition of claim 1, wherein the archaeal MCM is from or homologous to MCM found in *Pyrococcus species* or *Thermoccocus species*.

4. The composition of claim 1, wherein the at least one archaeal polypeptide is PCNA.

5. The composition of claim 1, wherein the at least one archaeal polypeptide is RFC-P38.

6. The composition of claim 1, wherein the at least one archaeal polypeptide is RFC-P55.

7. The composition of claim 1, wherein the at least one archaeal polypeptide is RFA.

8. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 2.

9. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 3.

10. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 4.

11. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 5.

12. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 6.

13. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 7.

14. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase 8.

15. The composition of claim 1, wherein the at least one archaeal polypeptide is helicase dna2.

16. The composition of claim 1, wherein the at least one archaeal polypeptide is CDC6.

17. The composition of claim 1, wherein the at least one archaeal polypeptide is FEN-1.

18. The composition of claim 1, wherein the at least one archaeal polypeptide is ligase.

19. The composition of claim 1, wherein the at least one archaeal polypeptide is dUTPase.

20. The composition of claim 1, further comprising a polymerase.

21. The composition of claim 20, wherein the polymerase is a thermostable polymerase.

22. The composition of claim 21, wherein the polymerase is Pfu polymerase.

23. The composition of claim 21, wherein the polymerase is *P. furiosus* pol II.

24. The composition of claim 20, further comprising a second polymerase.

25. The composition of claim 24, wherein the second polymerase is a thermostable polymerase.

26. he composition of claim 25, wherein the second polymerase lacks 3' to 5' exonuclease activity.

27. The composition of claim 26, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

28. The composition of claim 26, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

29. A composition for enhancing polymerase reactions comprising an archaeal CDC6 and at least one archaeal polypeptide selected from the

group consisting of PCNA, RFC-P38, RFC-P55, RFA, FEN-1, ligase, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, helicase 8, and dUTPase.

30. The composition of claim 29, wherein the at least one archaeal polypeptide is PCNA.

31. The composition of claim 29, wherein the at least one archaeal polypeptide is RFC-P38.

32. The composition of claim 29, wherein the at least one archaeal polypeptide is RFC-P55.

33. The composition of claim 29, wherein the at least one archaeal polypeptide is RFA.

34. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 2.

35. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 3.

36. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 4.

37. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 5.

38. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 6.

39. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 7.

40. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase 8.

41. The composition of claim 29, wherein the at least one archaeal polypeptide is helicase dna2.

42. The composition of claim 29, wherein the at least one archaeal polypeptide is FEN-1.

43. The composition of claim 29, wherein the at least one archaeal polypeptide is ligase.

44. The composition of claim 29, wherein the at least one archaeal polypeptide is dUTPase.

45. The composition of claim 29, further comprising a polymerase.

46. The composition of claim 45, wherein the polymerase is a thermostable polymerase.

47. The composition of claim 46, wherein the polymerase is Pfu polymerase.

48. The composition of claim 46, wherein the polymerase is *P. furiosus* pol II.

49. The composition of claim 45, further comprising a second polymerase.

50. The composition of claim 49, wherein the second polymerase is thermostable.

51. The composition of claim 50, wherein the second polymerase lacks 3' to 5' exonuclease activity.

52. The composition of claim 51, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

53. The composition of claim 51, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

54. A composition for enhancing nucleic acid polymerase reactions comprising an archaeal FEN-1 and at least one archaeal polypeptide selected from the group consisting of PCNA, RFC-P38, RFC-P55, RFA, ligase, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, helicase 8, and dUTPase.

55. The composition of claim 54, wherein the at least one archaeal polypeptide is PCNA.

56. The composition of claim 54, wherein the at least one archaeal polypeptide is RFC-P38.

57. The composition of claim 54, wherein the at least one archaeal polypeptide is RFC-P55.

58. The composition of claim 54, wherein the at least one archaeal polypeptide is RFA.

59. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 2.

60. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 3.

61. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 4.

62. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 5.

63. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 6.

64. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 7.

65. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase 8.

66. The composition of claim 54, wherein the at least one archaeal polypeptide is helicase dna2.

67. The composition of claim 54, wherein the at least one archaeal polypeptide is ligase.

68. The composition of claim 54, wherein the at least one archaeal polypeptide is dUTPase.

69. The composition of claim 54, further comprising a polymerase.

70. The composition of claim 69, wherein the polymerase is a thermostable polymerase.

71. The composition of claim 70, wherein the polymerase is Pfu polymerase.

72. The composition of claim 70, wherein the polymerase is *P. furiosus* pol II.

73. The composition of claim 69, further comprising a second polymerase.

74. The composition of claim 73, wherein the second polymerase is a thermostable polymerase.

75. The composition of claim 74, wherein the second polymerase lacks 3' to 5' exonuclease activity.

76. The composition of claim 75, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

77. The composition of claim 75, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

78. A composition for enhancing nucleic acid polymerase reactions comprising an archaeal RFA and at least one archaeal polypeptide selected from the group consisting of PCNA, RFC-P38, RFC-P55, FEN-1, ligase, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, helicase 8, and dUTPase.

79. The composition of claim 78, wherein the archaeal RFA is from or hologous to the RFA found in *Pyrococcus furiosus*.

80. The composition of claim 78, wherein the archaeal RFA is from or homologous to the RFA found in *Pyrococcus species* or *Thermoccocus species*.

81. The composition of claim 78, wherein the at least one archaeal polypeptide is PCNA.

82. The composition of claim 78, wherein the at least one archaeal polypeptide is RFC-P38.

83. The composition of claim 78, wherein the at least one archaeal polypeptide is RFC-P55.

84. The composition of claim 78, wherein the at least one archaeal polypeptide is FEN-1.

85. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 2.

86. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 3.

87. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 4.

88. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 5.

89. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 6.

90. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 7.

91. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase 8.

92. The composition of claim 78, wherein the at least one archaeal polypeptide is helicase dna2.

93. The composition of claim 78, wherein the at least one archaeal polypeptide is ligase.

94. The composition of claim 78, wherein the at least one archaeal polypeptide is dUTPase.

95. The composition of claim 78, further comprising a polymerase.

96. The composition of claim 95, wherein the polymerase is a thermostable polymerase.

97. The composition of claim 96, wherein the polymerase is Pfu polymerase.

98. The composition of claim 96, wherein the polymerase is *P. furiosus* pol II.

99. The composition of claim 95, further comprising a second polymerase.

100. The composition of claim 99, wherein the second polymerase is a thermostable polymerase.

101. The composition of claim 100, wherein the second polymerase lacks 3' to 5' exonuclease activity.

102. The composition of claim 101, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

103. The composition of claim 101, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

104. A composition for enhancing nucleic acid polymerase reactions comprising an archaeal ligase and at least one archaeal polypeptide selected from the group consisting of PCNA, RFC-P38, RFC-P55, RFA, CDC6, FEN-1, dUTPase, MCM, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, helicase 8, and dUTPase.

105. The composition of claim 104, wherein the at least one archaeal polypeptide is PCNA.

106. The composition of claim 104, wherein the at least one archaeal polypeptide is RFC-P38.

107. The composition of claim 104, wherein the at least one archaeal polypeptide is RFC-P55.

108. The composition of claim 104, wherein the at least one archaeal polypeptide is RFA.

109. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 2.

110. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 3.

111. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 4.

112. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 5.

113. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 6.

114. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 7.

115. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase 8.

116. The composition of claim 104, wherein the at least one archaeal polypeptide is helicase dna2.

117. The composition of claim 104, wherein the at least one archaeal polypeptide is CDC6.

118. The composition of claim 104, wherein the at least one archaeal polypeptide is FEN-1.

119. The composition of claim 104, wherein the at least one archaeal polypeptide is MCM.

120. The composition of claim 104, wherein the at least one archaeal polypeptide is dUTPase.

121. The composition of claim 104, further comprising a polymerase.

122. The composition of claim 121, wherein the polymerase is a thermostable polymerase.

123. The composition of claim 122, wherein the polymerase is Pfu polymerase.

124. The composition of claim 122, wherein the polymerase is *P. furiosus* pol II.

125. The composition of claim 121, further comprising a second polymerase.

126. The composition of claim 125, wherein the second polymerase is a thermostable polymerase.

127. The composition of claim 126, wherein the second polymerase lacks 3' to 5' exonuclease activity.

128. The composition of claim 127, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

129. The composition of claim 127, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

130. A composition for enhancing nucleic acid polymerase reactions comprising at least one archaeal accessory factor selected from the group consisting of MCM, RFA, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, and helicase dna2.

131. The composition of claim 130, wherein the at least one archaeal accessory factor is from or homologous to an accessory factor found in *Pyrococcus furiosus*.

132. The composition of claim 130, wherein the at least one archaeal accessory factor is from or homologous to an accessory factor found in *Pyrococcus species* or *Thermoccocus species*.

133. The composition of claim 130, wherein the at least one archaeal accessory factor comprises MCM.

134. The composition of claim 130, wherein the at least one archaeal accessory factor comprises RFA.

135. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase 2.

136. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase 3.

137. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase 4.

138. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase 5.

139. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase 6.

140. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase 7.

141. The composition of claim 130, wherein the at least one archaeal accessory factor comprises helicase dna2.

142. The composition of claim 130, further comprising a polymerase.

143. The composition of claim 142, wherein the polymerase is thermostable.

144. The composition of claim 143, wherein the polymerase is Pfu polymerase.

145. The composition of claim 143, wherein the polymerase *P. furiosus* pol II.

146. The composition of claim 142, further comprising a second polymerase.

147. The composition of claim 146, wherein the second polymerase is thermostable.

148. The compostion of claim 147, wherein the second polymerase lacks 3' to 5' nuclease activity.

149. The composition of claim 148, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

150. The composition of claim 148, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

151. A composition for enhancing nucleic acid polymerase reactions comprising archaeal CDC6, archaeal MCM, and at least one polymerase.

152. The composition of claim 151, wherein the CDC6 and MCM are from or homologous to the CDC6 and MCM found in *Pyrococcus species or Thermococcus species*.

153. The composition of claim 151, wherein the CDC6 and MCM are from or homologous to the CDC6 and MCM found in *Pyrococcus furiosus*.

154. The composition of claim 151, wherein the polymerase is Pfu polymerase.

155. The composition of claim 151, wherein the polymerase is *P. furiosus* pol II.

156. The composition of claim 151, further comprising a second polymerase.

157. The composition of claim 156, wherein the second polymerase is thermostable.

158. The composition of claim 157, wherein the second polymerase lacks 3' to 5' exonuclease activity.

159. The composition of claim 158, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

160. The composition of claim 158, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

161. The composition of claim 151, further comprising an archaeal dUTPase.

162. A composition for enhancing nucleic acid polymerase reactions comprising archaeal PCNA, archaeal FEN-1, and at least one polymerase.

163. The composition of claim 162, wherein the PCNA and FEN-1 are from or homologous to the PCNA and FEN-1 found in *Pyrococcus species or Thermococcus species*.

164. The composition of claim 162, wherein the PCNA and FEN-1 are from or homologous to the PCNA and FEN-1 found in *Pyrococcus furiosus*.

165. The composition of claim 162, wherein the polymerase is Pfu polymerase.

166. The composition of claim 162, wherein the polymerase is *P. furiosus* pol II.

167. The composition of claim 162, further comprising a second polymerase.

168. The composition of claim 167, wherein the second polymerase is thermostable.

169. The composition of claim 168, wherein the second polymerase lacks 3' to 5' exonuclease activity.

170. The composition of claim 169, wherein the second polymerase is Taq, Tfl, Tbr, or Tth polymerase.

171. The composition of claim 169, wherein the second polymerase is Stoffel, Tru, Tca, or Tfil polymerase.

172. The composition of claim 162, further comprising an archaeal dUTPase.

173. An isolated and purified polynucleotide encoding an archaeal RFA comprising: (a) a polynucleotide comprising the nucleotide sequence set forth in Figure 16 (SEQ ID NO:) or the nucleotide sequence of Figure 16 starting with nucleotide 7; (b) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 17 (SEQ ID NO:) or the amino acid sequence of Figure 17 starting with amino acid 3; or (c) an analog or degenerate variant of (a) or (b).The polynucleotide of claim 173, wherein the polynucleotide is genomic DNA.

174. The polynucleotide of claim 173, wherein the polynucleotide is cDNA.

175. The polynucleotide of claim 173, wherein the polynucleotide is mRNA.

176. The polynucleotide of claim 173, wherein the polynucleotide is fully or partially synthetic.

177. An isolated and purified polynucleotide encoding an archaeal MCM comprising: (a) the nucleotide sequence set forth in Figure 45 (SEQ ID NO:) or Figure 55 (SEQ ID NO:); (b) a polynucleotide sequence encoding an amino acid sequence comprising the amino acid sequence set forth in Figures 46 (SEQ ID NO:) or 47 (SEQ ID NO:); or (c) an analog or a degenerate variant of (a) or (b).

178. The polynucleotide of claim 178, wherein the polynucleotide is genomic DNA.

179. The polynucleotide of claim 178, wherein the polynucleotide is cDNA.

180. The polynucleotide of claim 178, wherein the polynucleotide is mRNA.

181. The polynucleotide of claim 178, wherein the polynucleotide is fully or partially synthetic.

182. An isolated and purified polynucleotide encoding an archaeal helicase, wherein the polynucleotide is selected from the group consisting of: (a) a

polynucleotide comprising the nucleotide sequence set forth in Figure 22 (SEQ ID NO:); (b) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 29 (SEQ ID NO:); (c) a polynucleotide comprising the nucleotide sequence set forth in Figure 23 (SEQ ID NO:); (d) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 30 (SEQ ID NO:); (e) a polynucleotide comprising the nucleotide sequence set forth in Figure 24 (SEQ ID NO:); (f) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 31 (SEQ ID NO:); (g) a polynucleotide comprising the nucleotide sequence set forth in Figure 25 (SEQ ID NO:); (h) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 32 (SEQ ID NO:); (i) a polynucleotide comprising the nucleotide sequence set forth in Figure 26 (SEQ ID NO:); (j) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 33 (SEQ ID NO:); (k) a polynucleotide comprising the nucleotide sequence set forth in Figure 27 (SEQ ID NO:); (l) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 34 (SEQ ID NO:); (m) a polynucleotide comprising the nucleotide sequence set forth in Figure 28 (SEQ ID NO:); (n) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 35 (SEQ ID NO:); (o) a polynucleotide comprising the nucleotide sequence set forth in Figure 40 (SEQ ID NO:); (p) a polynucleotide encoding an amino acid sequence comprising the amino acid sequence set forth in Figure 41 (SEQ ID NO:); and (o) an analog or degenerate variant of (a) through (p).

183. The polynucleotide of claim 183, wherein the polynucleotide is genomic DNA.

184. The polynucleotide of claim 183, wherein the polynucleotide is cDNA.

185. The polynucleotide of claim 183, wherein the polynucleotide is mRNA.

186. The polynucleotide of claim 183, wherein the polynucleotide is fully or partially synthetic.

187. An isolated and purified archaeal polypeptide selected from the group consisting of MCM, RFA, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, and helicase 7.

188. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 46 or 47 (SEQ ID NO:).

189. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 17 (SEQ ID NO:) or the amino acid sequence of Figure 17 starting with amino acid 3.

190. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 29 (SEQ ID NO:).

191. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 30 (SEQ ID NO:).

192. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 31 (SEQ ID NO:).

193. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 32 (SEQ ID NO:).

194. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 33 (SEQ ID NO:).

195. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 34 (SEQ ID NO:).

196. The isolated and purified archaeal polypeptide of claim 188 comprising the amino acid sequence of Figure 35 (SEQ ID NO:).

197. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 1 in the nucleic acid polymerase reaction.

198. The method of claim 198 comprising employing the composition of claim 1 in a nucleic acid synthesis reaction.

199. The method of claim 198 comprising employing the composition of claim 1 in a nucleic acid amplification reaction.

200. The method of claim 198 comprising employing the composition of claim 1 when mutagenizing a nucleic acid.

201. The method of claim 198 comprising employing the composition of claim 1 when labeling a nucleic acid.

202. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 29 in a nucleic acid polymerase reaction.

203. The method of claim 203 comprising employing the composition of claim 29 in a nucleic acid synthesis reaction.

204. The method of claim 203 comprising employing the composition of claim 29 in a nucleic acid amplification reaction.

205. The method of claim 203 comprising employing the composition of claim 29 when mutagenizing a nucleic acid.

206. The method of claim 203 comprising employing the composition of claim 29 when labeling a nucleic acid.

207. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 54 in the nucleic acid polymerase reaction.

208. The method of claim 208 comprising employing the composition of claim 54 in a nucleic acid synthesis reaction.

209. The method of claim 208 comprising employing the composition of claim 54 in a nucleic acid amplification reaction.

210. The method of claim 208 comprising employing the composition of claim 54 when mutagenizing a nucleic acid.

211. The method of claim 208 comprising employing the composition of claim 54 when labeling a nucleic acid.

212. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 78 in the nucleic acid polymerase reaction.

213. The method of claim 213 comprising employing the composition of claim 78 in a nucleic acid synthesis reaction.

214. The method of claim 213 comprising employing the composition of claim 78 in a nucleic acid amplification reaction.

215. The method of claim 213 comprising employing the composition of claim 78 when mutagenizing a nucleic acid.

216. The method of claim 213 comprising employing the composition of claim 78 when labeling a nucleic acid.

217. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 104 in a nucleic acid polymerase reaction.

218. The method of claim 218 comprising employing the composition of claim 104 in a nucleic acid synthesis reaction.

219. The method of claim 218 comprising employing the composition of claim 104 in a nucleic acid amplification reaction.

220. The method of claim 218 comprising employing the composition of claim 104 when mutagenizing a nucleic acid.

221. The method of claim 218 comprising employing the composition of claim 104 when labeling a nucleic acid.

222. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 130 in the nucleic acid polymerase reaction.

223. The method of claim 223 comprising employing the composition of claim 130 in a nucleic acid synthesis reaction.

224. The method of claim 223 comprising employing the composition of claim 130 in a nucleic acid amplification reaction.

225. The method of claim 223 comprising employing the composition of claim 130 when mutagenizing a nucleic acid.

226. The method of claim 223 comprising employing the composition of claim 130 when labeling a nucleic acid.

227. A method of enhancing a nucleic acid polymerase reaction comprising employing the composition of claim 151 in a nucleic acid polymerase reaction.

228. The method of claim 228 comprising employing the composition of claim 151 in a nucleic acid synthesis reaction.

229. The method of claim 228 comprising employing the composition of claim 151 in a nucleic acid amplification reaction.

230. The method of claim 228 comprising employing the composition of claim 151 when mutagenizing a nucleic acid.

231. The method of claim 228 comprising employing the composition of claim 151 when labeling a nucleic acid.

232. A method for increasing stability of nucleic acid duplexes in a nucleic acid polymerase reaction comprising adding to the polymerase reaction at least one accessory factor selected from the group consisting of archaeal MCM, archaeal CDC6, and archaeal FEN-1.

233. The method of claim 233, wherein the MCM is from or homologous to the MCM found in *Pyrococcus species* or *Thermococcus species.*

234. The method of claim 233, wherein the MCM is from or homologous to the MCM found in *Pyrococcus furiosus.*

235. The method of claim 233, wherein the CDC6 is from or homologous to the CDC6 found in *Pyrococcus species* or *Thermococcus species.*

236. The method of claim 233, wherein the CDC6 is from or homologous to the CDC6 found in *Pyrococcus furiosus.*

237. The method of claim 233, wherein the FEN-1 is from or homologous to the FEN-1 found in *Pyrococcus species* or *Thermococcus species.*

238. The method of claim 233, wherein the FEN-1 is from or homologous to the FEN-1 found in *Pyrococcus furiosus..*

239. The method of claim 233, wherein the MCM, CDC6, and FEN-1 are from or homologous to the MCM, CDC6, and FEN-1 found in *Pyrococcus species* or *Thermococcus species.*

240. The method of claim 233, wherein the MCM, CDC6, and FEN-1 are from or homologous to the MCM, CDC6, and FEN-1 found in *Pyrococcus furiosus.*

241. A method for increasing stability of nucleic acid duplexes in a nucleic acid polymerase reaction comprising adding to the polymerase reaction an archaeal PCNA and at least one archaeal polypeptide selected from the group consisting of MCM, CDC6, FEN-1, RFC-P38, RFC-P55, RFA, dUTPase, ligase, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, and helicase 8.

242. The method of claim 242, wherein the archaeal PCNA is from or homologous to the PCNA found in *Pyrococcus species* or *Thermococcus species*.

243. The method of claim 242, wherein the at least one archaeal polypeptide is from or homologous to an archaeal polypeptide found in *Pyrococcus species* or *Thermococcus species*.

244. The method of claim 242, wherein the archaeal PCNA and the at least one archaeal polypeptide are from or homologous to the PCNA and the at least one archaeal polypeptide found in *Pyrococcus furiosus*.

245. A method for enhancing an exonuclease reaction comprising adding to the exonuclease reaction an archaeal PCNA and at least one archaeal polypeptide selected from the group consisting of MCM, CDC6, FEN-1, RFC-P38, RFC-P55, RFA, dUTPase, ligase, helicase dna2, helicase 2, helicase 3, helicase 4, helicase 5, helicase 6, helicase 7, and helicase 8.

246. The method of claim 246, wherein the archaeal PCNA is from or homologous to the PCNA found in *Pyrococcus species* or *Thermococcus species*.

247. The method of claim 246, wherein the at least one archaeal polypeptide is from or homologous to an archaeal polypeptide found in *Pyrococcus species* or *Thermococcus species*.

248. The method of claim 246, wherein the archaeal PCNA and the at least one archaeal polypeptide are from or homologous to the PCNA and the at least one archaeal polypeptide found in *Pyrococcus furiosus*.

249. The method of claim 246, wherein the MCM is from or homologous to the MCM found in *Pyrococcus furiosus*.

250. The method of claim 246, wherein the MCM is from or homologous to the MCM found in *Pyrococcus species* or *Thermococcus species*.

251. The method of claim 246, wherein the CDC6 is from or homologous to the CDC6 found in *Pyrococcus furiosus*.

252. The method of claim 246, wherein the CDC6 is from or homologous to the CDC6 found in *Pyrococcus species* or *Thermococcus species*.

253. The method of claim 246, wherein the FEN-1 is from or homologous to the FEN-1 found in *Pyrococcus furiosus*.

254. The method of claim 246, wherein the FEN-1 is from or homologous to the FEN-1 found in *Pyrococcus species* or *Thermococcus species*.

255. The method of claim 246, wherein the MCM, CDC6, and FEN-1 are from or homologous to the MCM, CDC6, and FEN-1 found in *Pyrococcus furiosus*.

256. The method of claim 246, wherein the MCM, CDC6, and FEN-1 are from or homologous to the MCM, CDC6, and FEN-1 found in *Pyrococcus species* or *Thermococcus species*.

257. A method for producing a *Pyrococcus furiosus* MCM polypeptide comprising:

expressing the polynucleotide of the vector of claim 304 in a host cell; and

purifying the expressed MCM polypeptide.

258. The method of claim 258, wherein the host cell is a prokaryotic cell.

259. The method of claim 258, wherein the host cell is a eukaryotic cell.

260. A recombinant MCM polypeptide produced by the method of claim 258.

261. A method for producing a *Pyrococcus furiosus* RFA polypeptide comprising:

expressing the polynucleotide of the vector of claim 313 in a host cell; and

purifying the expressed RFA polypeptide.

262. The method of claim 262, wherein the host cell is a prokaryotic cell.

263. The method of claim 262, wherein the host cell is a eukaryotic cell.

264. A recombinant RFA polypeptide produced by the method of claim 262.

265. A method for producing a *Pyrococcus furiosus* helicase polypeptide comprising:

expressing the polynucleotide of the vector of claim 322 in a host cell; and

purifying the expressed helicase polypeptide.

266. The method of claim 266, wherein the host cell is a prokaryotic cell.

267. The method of claim 266, wherein the host cell is a eukaryotic cell.

268. A recombinant helicase polypeptide produced by the method of claim 266.

269. An isolated and purified archaeal MCM polypeptide.

270. The polypeptide of claim 270, wherein the polypeptide is from or homologous to the MCM found in *Pyrococcus species* or *Thermococcus species*.

271. The polypeptide of claim 270, wherein the polypeptide is from or homologous to the MCM found in *Pyrococcus furiosus*.

272. An isolated and purified archaeal RFA polypeptide.

273. The polypeptide of claim 273, wherein the polypeptide is from or homologous to the RFA found in *Pyrococcus species* or *Thermococcus species*.

274. The polypeptide of claim 273, wherein the polypeptide is from or homologous to the RFA found in *Pyrococcus furiosus*.

275. A kit comprising the composition of claim 1.

276. The kit of claim 276 for use in enhancing nucleic acid polymerase reactions.

277. The kit of claim 276 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

278. The kit of claim 276 for use in labeling or detecting nucleic acids.

279. A kit comprising the composition of claim 29.

280. The kit of claim 280 for use in enhancing nucleic acid polymerase reactions.

281. The kit of claim 280 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

282. The kit of claim 280 for use in labeling or detecting nucleic acids.

283. A kit comprising the composition of claim 54.

284. The kit of claim 284 for use in enhancing nucleic acid polymerase reactions.

285. The kit of claim 284 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

286. The kit of claim 284 for use in labeling or detecting nucleic acids.

287. A kit comprising the composition of claim 78.

288. The kit of claim 288 for use in enhancing nucleic acid polymerase reactions.

289. The kit of claim 288 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

290. The kit of claim 288 for use in labeling or detecting nucleic acids.

291. A kit comprising the composition of claim 104.

292. The kit of claim 292 for use in enhancing nucleic acid polymerase reactions.

293. The kit of claim 292 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

294. The kit of claim 292 for use in labeling or detecting nucleic acids.

295. A kit comprising the composition of claim 130.

296. The kit of claim 296 for use in enhancing nucleic acid polymerase reactions.

297. The kit of claim 296 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

298. The kit of claim 296 for use in labeling or detecting nucleic acids.

299. A kit comprising the composition of claim 151.

300. The kit of claim 300 for use in enhancing nucleic acid polymerase reactions.

301. The kit of claim 300 for use in synthesizing, amplifying, or mutagenizing nucleic acids.

302. The kit of claim 300 for use in labeling or detecting nucleic acids.

303. A vector comprising the polynucleotide of claim 178.

304. The vector of claim 304, wherein the vector is a plasmid.

305. The vector of claim 304, wherein the vector is a bacteriophage.

306. The vector of claim 304, wherein the vector is a retrovirus.

307. The vector of claim 304, wherein the vector is an adenovirus.

308. The vector of claim 304, wherein the vector is a baculovirus.

309. A host cell comprising the vector of claim 304.

310. The host cell of claim 310, wherein the host cell is a prokaryotic cell.

311. The host cell of claim 310, wherein the host cell is a eukaryotic cell.

312. A vector comprising the polynucleotide of claim 173.

313. The vector of claim 313, wherein the vector is a plasmid.

314. The vector of claim 313, wherein the vector is a bacteriophage.

315. The vector of claim 313, wherein the vector is a retrovirus.

316. The vector of claim 313, wherein the vector is an adenovirus.

317. The vector of claim 313, wherein the vector is a baculovirus.

318. A host cell comprising the vector of claim 313.

319. The host cell of claim 319, wherein the host cell is a prokaryotic cell.

320. The host cell of claim 319, wherein the host cell is a eukaryotic cell.

321. A vector comprising the polynucleotide of claim 183.

322. The vector of claim 322, wherein the vector is a plasmid.

323. The vector of claim 322, wherein the vector is a bacteriophage.

324. The vector of claim 322, wherein the vector is a retrovirus.

325. The vector of claim 322, wherein the vector is an adenovirus.

326. The vector of claim 322, wherein the vector is a baculovirus.

327. A host cell comprising the vector of claim 322.

328. The host cell of claim 328, wherein the host cell is a prokaryotic cell.

329. The host cell of claim 328, wherein the host cell is a eukaryotic cell.

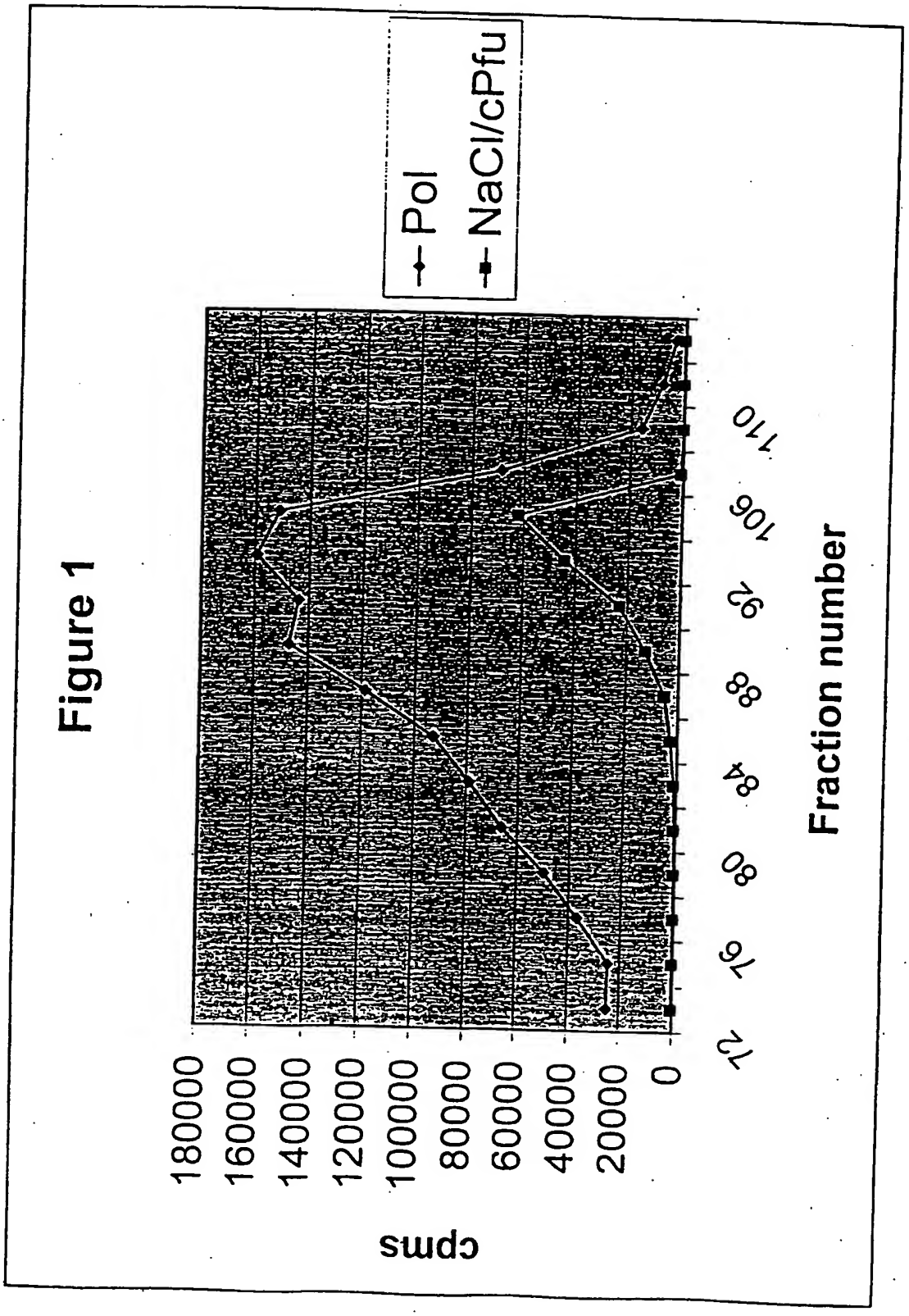
Pol
NaCl/cPfu
cpms
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
72
76
80
84
88
92
96
100
104
108
110
Fraction number

Figure 1

Figure 2

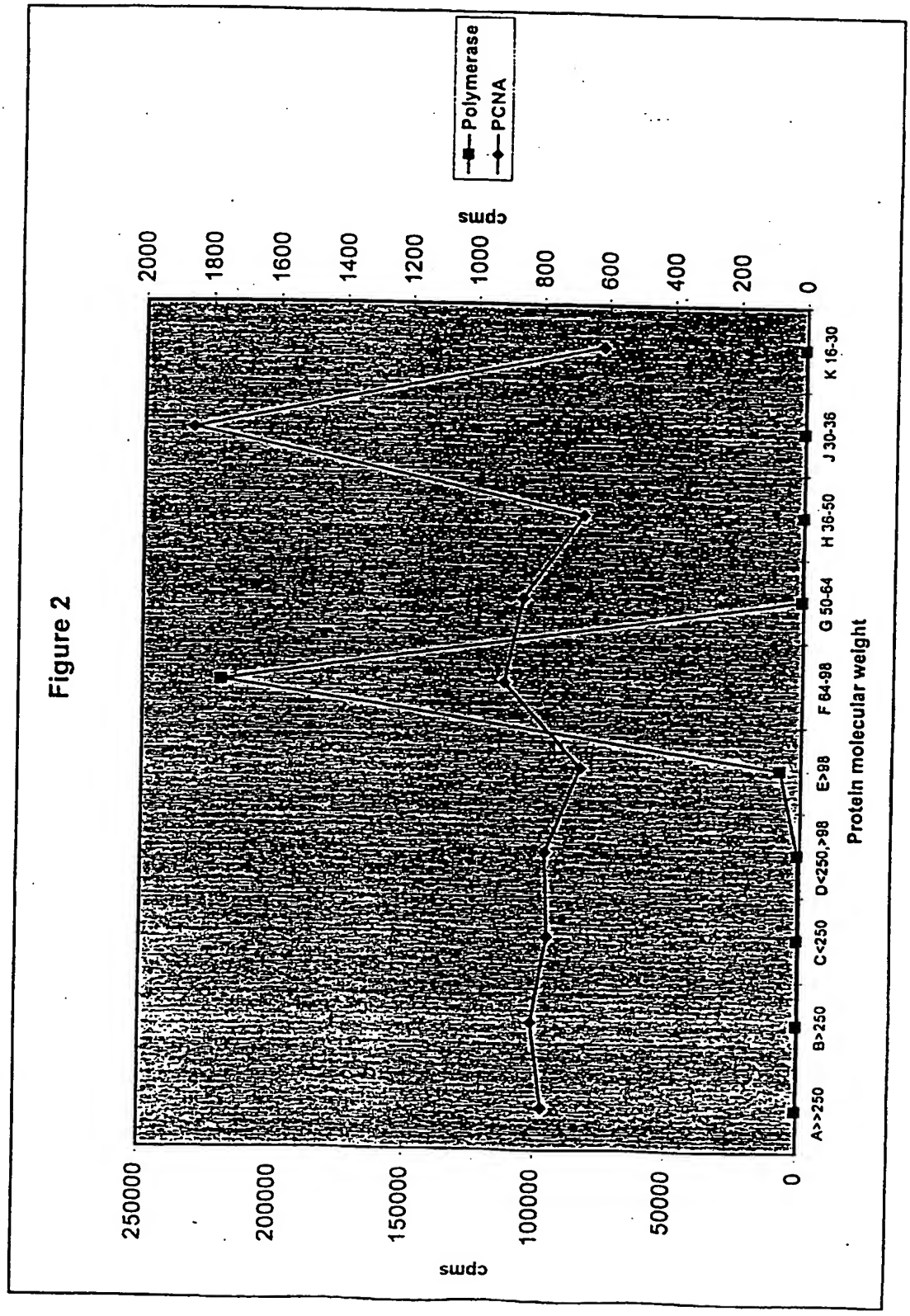
Polymerase
PCNA
cpms
2000
1800
1600
1400
1200
1000
800
600
400
200
0
250000
200000
150000
100000
50000
0
A>>250
B>250
C<250
D<250,>98
E>98
F 84-98
G 50-64
H 36-50
J 30-36
K 16-30
Protein molecular weight

Figure 3

ATGCCATTCGAAATAGTCTTTGAAGGTGCAAAAGAGTTTGCCCAACTTATAGACACCGCA
AGTAAGTTAATAGATGAGGCCGCGTTTAAAGTTACAGAAGATGGGATAAGCATGAGGGCC
ATGGATCCAAGTAGAGTTGTCCTGATTGACCTAAATCTCCCGTCAAGCATATTTAGCAAA
TATGAAGTTGTTGAACCAGAAACAATTGGAGTTAACATGGACCACCTAAAGAAGATCCTA
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ATAACAATTCAAGGAACTGCAACAAGAACATTTAGAGTTCCCCTAATAGATGTAGAAGAG
ATGGAAGTTGACCTCCCAGAACTTCCATTCACTGCAAAGGTTGTAGTTCTTGGAGAAGTC
CTAAAAGATGCTGTTAAAGATGCCTCTCTAGTGAGTGACAGCATAAAATTTATTGCCAGG
GAAATGAATTTATAATGAAGGCAGAGGGAGAAACCCAGGAAGTTGAGATAAAGCTAACT
CTTGAAGATGAGGGATTATTGGACATCGAGGTTCAAGAGGAGACAAAGAGCGCATATGGA
GTCAGCTATCTCTCCGACATGGTTAAAGGACTTGGAAAGGCCGATGAAGTTACAATAAAG
TTTGGAAATGAAATGCCCATGCAAATGGAGTATTACATTAGAGATGAAGGAAGACTTACA
TTCCTACTAGCCCCTAGGGTTGAGGAGTGA

Figure 4

MPFEIVFEGAKEFAQLIDTASKLIDEAAFKVTEDGISMRAMDPSRVVLID
LNLPSSIFSKYEVVEPETIGVNMDHLKKILKRGKAKDTLILKKGEENFLE
ITIQGTATRTFRVPLIDVEEMEVDLPELPFTAKVVVLGEVLKDAVKDASL
VSDSIKFIARENEFIMKAEGETQEVEIKLTLEDEGLLDIEVQEETKSAYG
VSYLSDMVKGLGKADEVTIKFGNEMPMQMEYYIRDEGRLTFLLAPRVEE*

Clamp Increases Processivity of *Pfu*

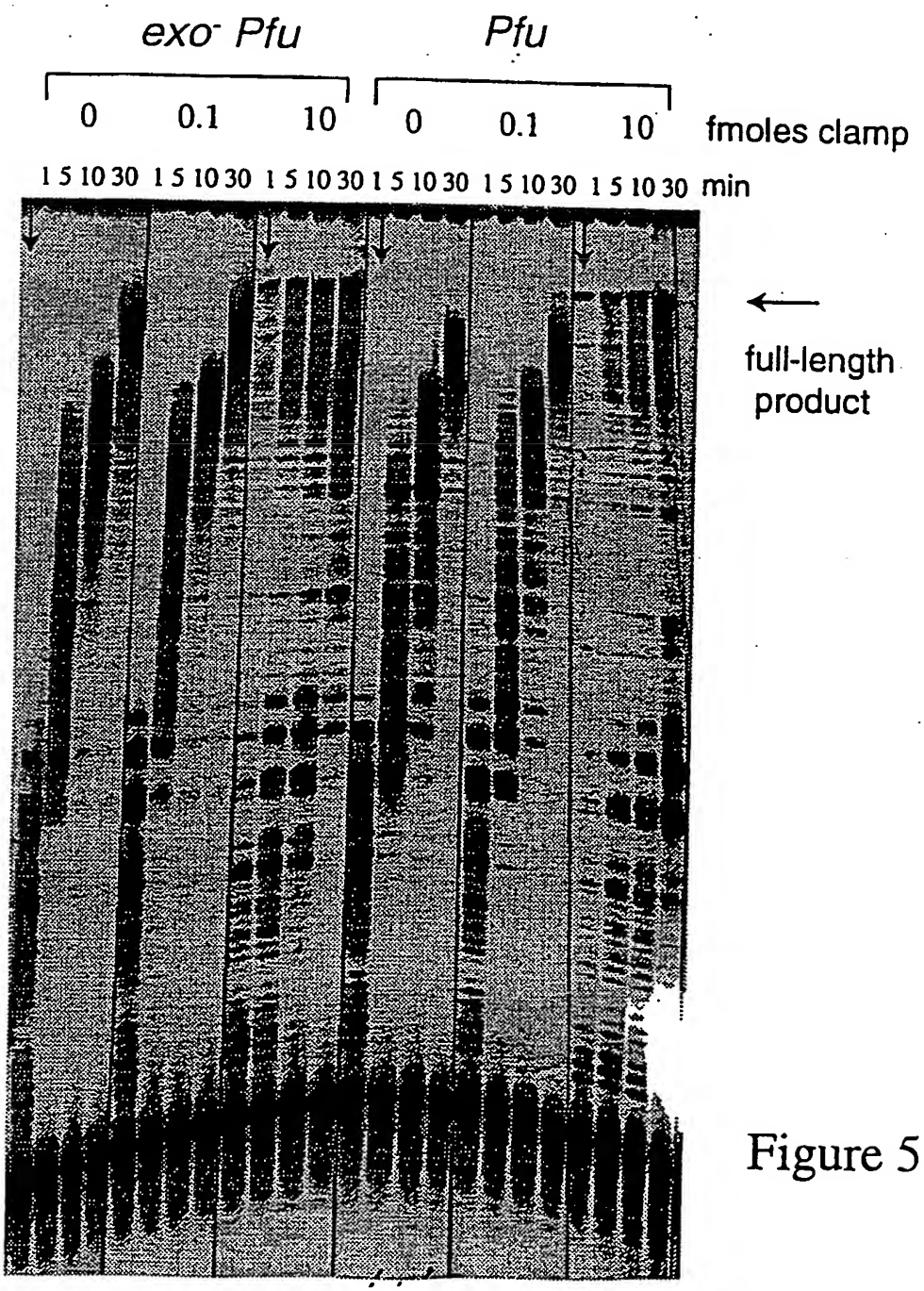
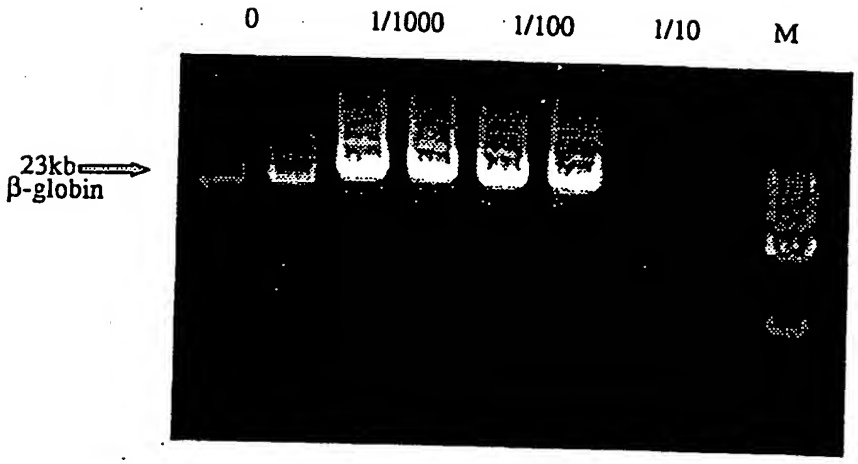


Figure 5.

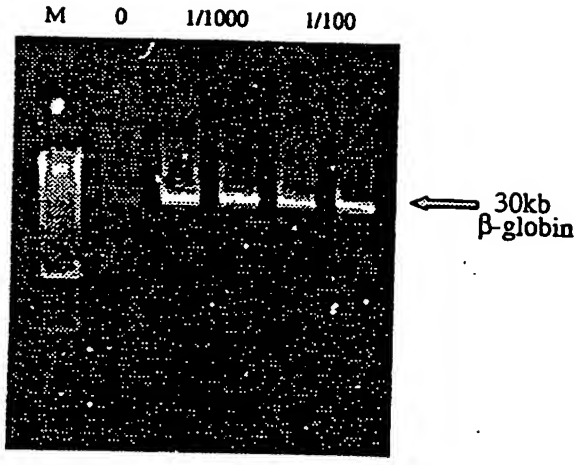
CLAMP



5U TPL + 1µl of clamp dilution / 50 µl PCR

Figure 6.

CLAMP



5U TPL + 1µl of clamp dilution / 50 µl PCR

Figure 7.

Figure 8

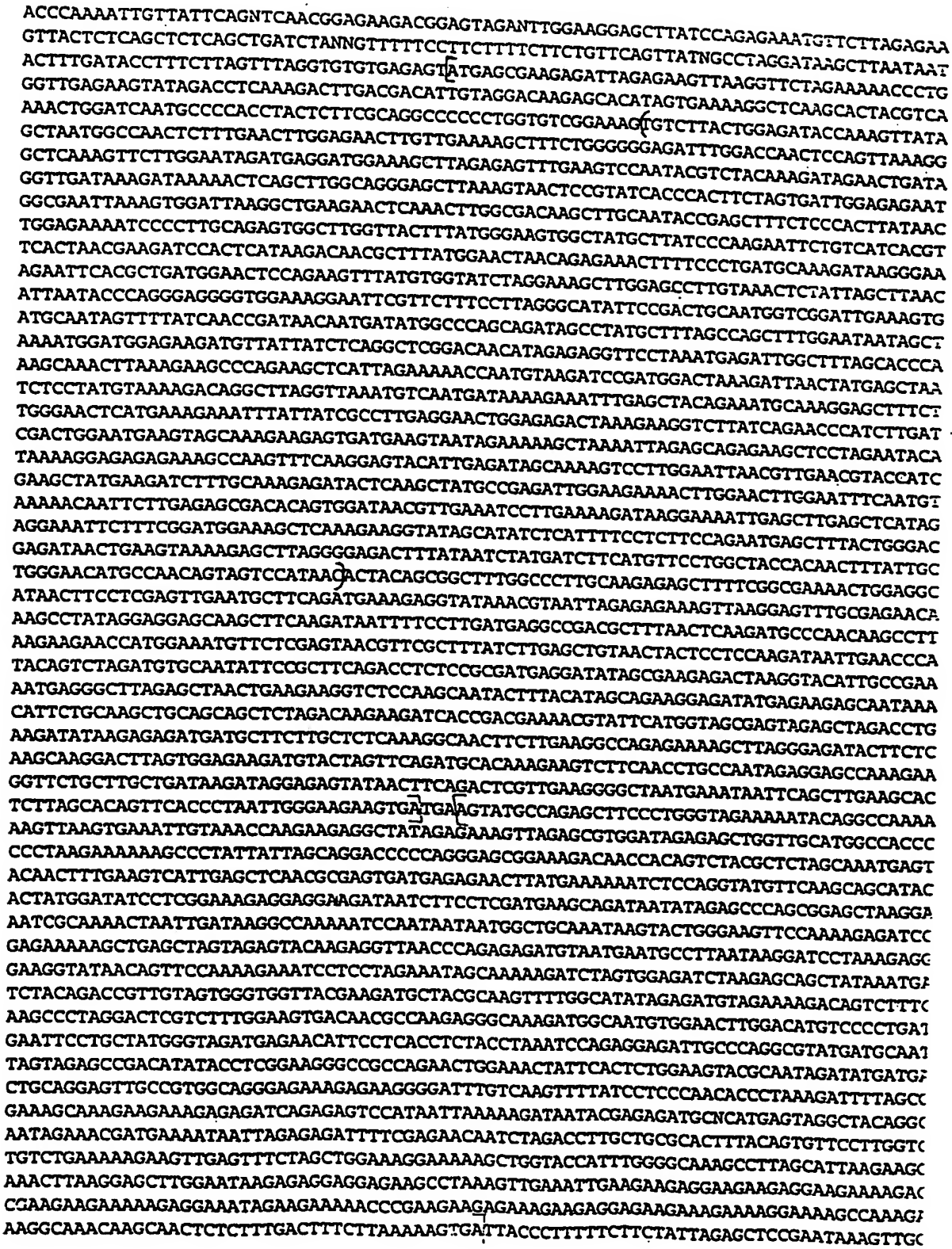


Figure 8 (cont.)

CCCTCTAATTTTTTCTATTGTCTCCTCCACATTAATCTTTACGAATTGGAATTCCTGCAGCCCGGGGGATCCACTAGTTC
TAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCATGG
TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAACCCGGAAGCATAAATTGTAAAC
CCNGGGGTGCCTAATGANTGANCTAACTCACATTAATTGCNTTGCGCTCACTGCCCGCTTTCCANTCGGGAAACCTGTCG
TGCCAGCTGCATTAATGAATCGGCCAACNCGCGGGGANAAGCGGTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCATG
ACTCGCTGCGCTCGGTCNTCGGCTGCGGCGAACGGTATCAGCTCATCAAAGGCGGTAATACGGTTATCCNCAAATCAGGG
GATAACGCAGGAAAAAACTTTNNACAAAAGGCNNCAAAAGGCGGAAACTAAAAGGCGCNTTCTGGGTTTTTCNTAGGCCC
NCCCCGANAACTCNAAAAATCAACNCATTCAAGTGGGAAACCAAAGAA

Figure 9

PKIVIQXNGEDGVXLEGAYPEKCS*RSYSQLSADLXFFLLFFCSVXA*DKLNNTLIPFLV*VCES(MSEEIREVKVLEKP
WVEKYRPQRLDDIVGQEHIVKRLKHYVKTGSMPHLLFAGPPGVGK[CLTGDTKVIANGQLFELGELVEKLSGGRFGPTPV
KGLKVLGIDEDGKLREFFEVQYVYKDRTDRLIKIKTQLGRELKVTPYHPLLVIGENGELKWIKAEELKLGDKLAIPSFLPL
ITGENPLAEWLGYFMGSGYAYPKNSVITFTNEDPLIRQRFMELTEKLFPDAKIRERIHADGTPEVYVVSRKAWSLVNSIS
LTLIPREGWKGIRSFLRAYSDCNGRIESDAIVLSTDNNDMAQQIAYALASFGIIAKMDGEDVIISGSDNIERFLNEIGFS
TQSKLKEAQKLIRKTNVRSDGLKINYELISYVKDRLRLNVNDKRNLSYRNAKELSWELMKEIYYRLEELERLKKVLSEPI
LIDWNEVAKKSDEVIEKAKIRAEKLLEYIKGERKPSFKEYIEIAKVLGINVERTIEAMKIFAKRYSSYAEIGRKLGTWNF
NVKTILESDTVDNVEILEKIRKIELELIEEILSDGKLKEGIAYLIFLFQNELYWDEITEVKELRGDFIIYDLHVPGYHNF
IAGNMPTVVHN]TTAALALARELFGENWRHNFLELNASDERGINVIREKVKEFARTKPIGGASFKIIFLDEADALTQDAQ
QALRRTMEMFSSNVRFILSCNYSSKIIEPIQSRCAIFRFRPLRDEDIAKRLRYIAENEGLELTEEGLQAILYIAEGDMRR
AINILQAAAALDKKITDENVFMVASRARPEDIREMMLLALKGNFLKAREKLREILLKQGLSGEDVLVQMHKEVFNLPIEE
PKKVLLADKIGEYNFRLVEGANEIIQLEALLAQFTLIGKK)**S(MPELPWVEKYRPKKLSEIVNQEEAIEKVRAWIESW
LHGHPPKKKALLLAGPPGSGKTTTVYALANEYNFEVIELNASDERTYEKISRYVQAAYTMDILGKRRKIIFLDEADNIEP
SGAKEIAKLIDKAKNPIIMAANKYWEVPKEIREKAELVEYKRLTQRDVMNALIRILKREGITVPKEILLEIAKRSSGDLR
AAINDLQTVVVGGYEDATQVLAYRDVEKTVFQALGLVFGSDNAKRAKMAWWNLDMSPDEFLLWVDENIPHLYLNPEEIAQ
AYDAISRADIYLGRAARTGNYSLWKYAIDMMTAGVAVAGRKRRGFVKFYPPNTLKILAESKEEREIRESIIKKIIREMXM
SRLQAIETMKIIREIFENNLDLAAHFTVFLGLSEKEVEFLAGKEKAGTIWGKALALRRKLKELGIREEEKPKVEIEEEEE
EEEKTEEEKEEIEEKPEEEKEEEKKEKEKPKKGKQATLFDFLKK)*LPFFFY*SSE*SWPSNFFYCLLHINLYELEFLQP
GGSTSSRAAATAVELQLLFPLVRVNFELGVIMVIAVSCVKLLSAHNSTQHTNPEA*IVNPGVPNX*XNSH*LXCAHCPLS
XRETCRASCINESANXRGXAVAYWALFRFLAHDSLRSVXGCGERYQLIKGGNTVIXKSGDNAGKNFXQKAXKGGN*KAXS
GFFXGPPRXLXKSTHSSGKPK

Figure 10

MPELPWVEKYRPKKLSEIVNQEEAIEKVRAWIESWLHGHPPKKKALLLAGPPGSGKTTTVYALANEYNFEVIELNASDER
TYEKISRYVQAAYTMDILGKRRKIIFLDEADNIEPSGAKEIAKLIDKAKNPIIMAANKYWEVPKEIREKAELVEYKRLTQ
RDVMNALIRILKREGITVPKEILLEIAKRSSGDLRAAINDLQTVVVGGYEDATQVLAYRDVEKTVFQALGLVFGSDNAKR
AKMAMWNLDMSPDEFLLWVDENIPHLYLNPEEIAQAYDAISRADIYLGRAARTGNYSLWKYAIDMMTAGVAVAGRKRRGF
VKFYPPNTLKILAESKEEREIRESIIKKIIREMXMSRLQAIETMKIIREIFENNLDLAAHFTVFLGLSEKEVEFLAGKEK
AGTIWGKALALRRKLKELGIREEEKPKVEIEEEEEEEEKTEEEKEEIEEKPEEEKKEKEKEKPKKGKQATLFDFLKK*

Figure 11

MSEEIREVKVLEKPWVEKYRPQRLDDIVGQEHIVKRLKHYVKTGSMPHLLFAGPPGVGKTTAALALARELFGENWRHNFL
ELNASDERGINVIREKVKEFARTKPIGGASFKIIFLDEADALTQDAQQALRRTMEMFSSNVRFILSCNYSSKIIEPIQSR
CAIFRFRPLRDEDIAKRLRYIAENEGLELTEEGLQAILYIAEGDMRRAINILQAAAALDKKITDENVFMVASRARPEDIR
EMMLLALKGNFLKAREKLREILLKQGLSGEDVLVQMHKEVFNLPIEEPKKVLLADKIGEYNFRLVEGANEIIQLEALLAQ
FTLIGKK**

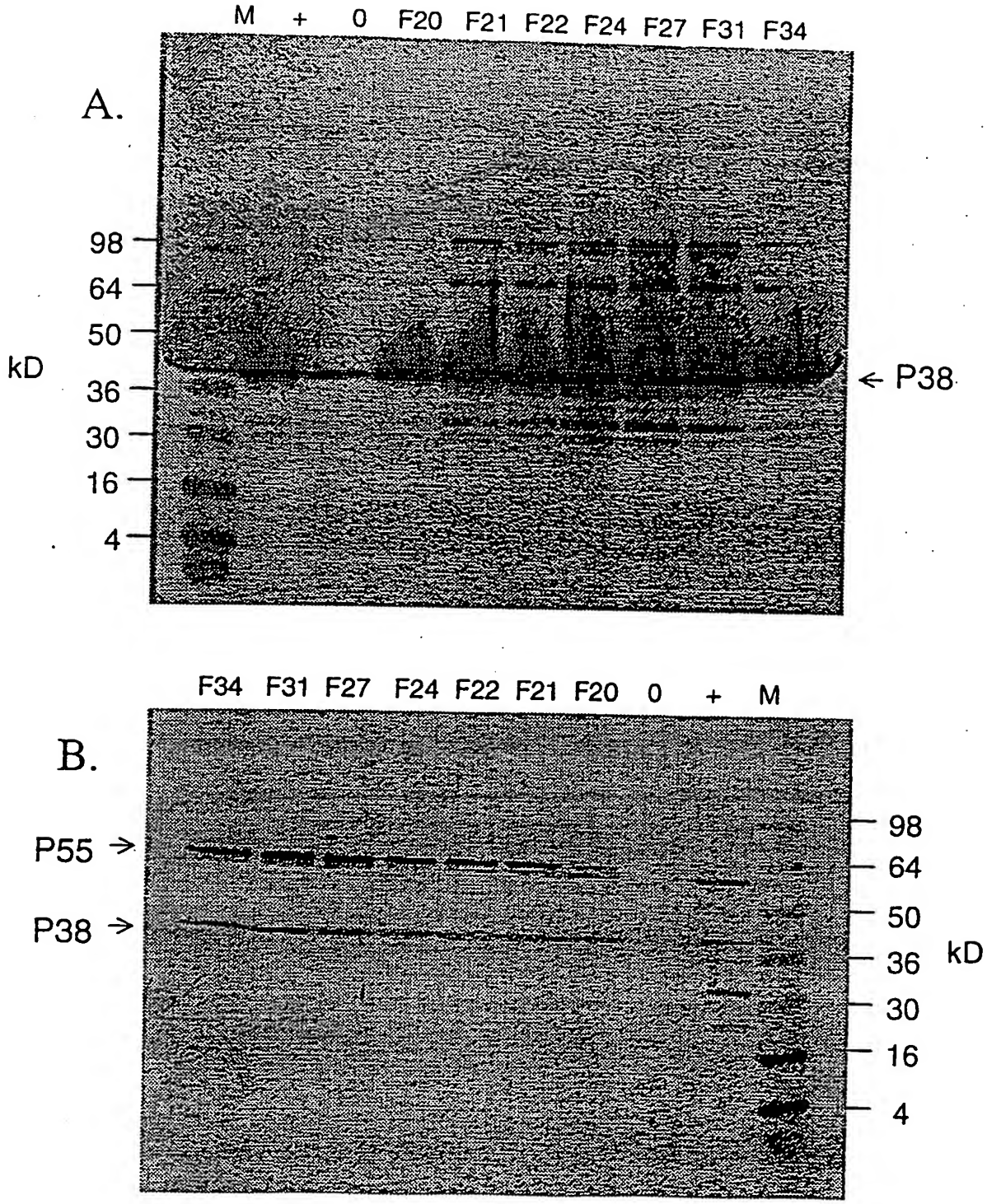

A.
M + 0 F20 F21 F22 F24 F27 F31 F34
98
64
50
kD
36
30
16
4
← P38
B.
F34 F31 F27 F24 F22 F21 F20 0 + M
P55 →
P38 →
98
64
50
36
30
16
4
kD

Figure 12

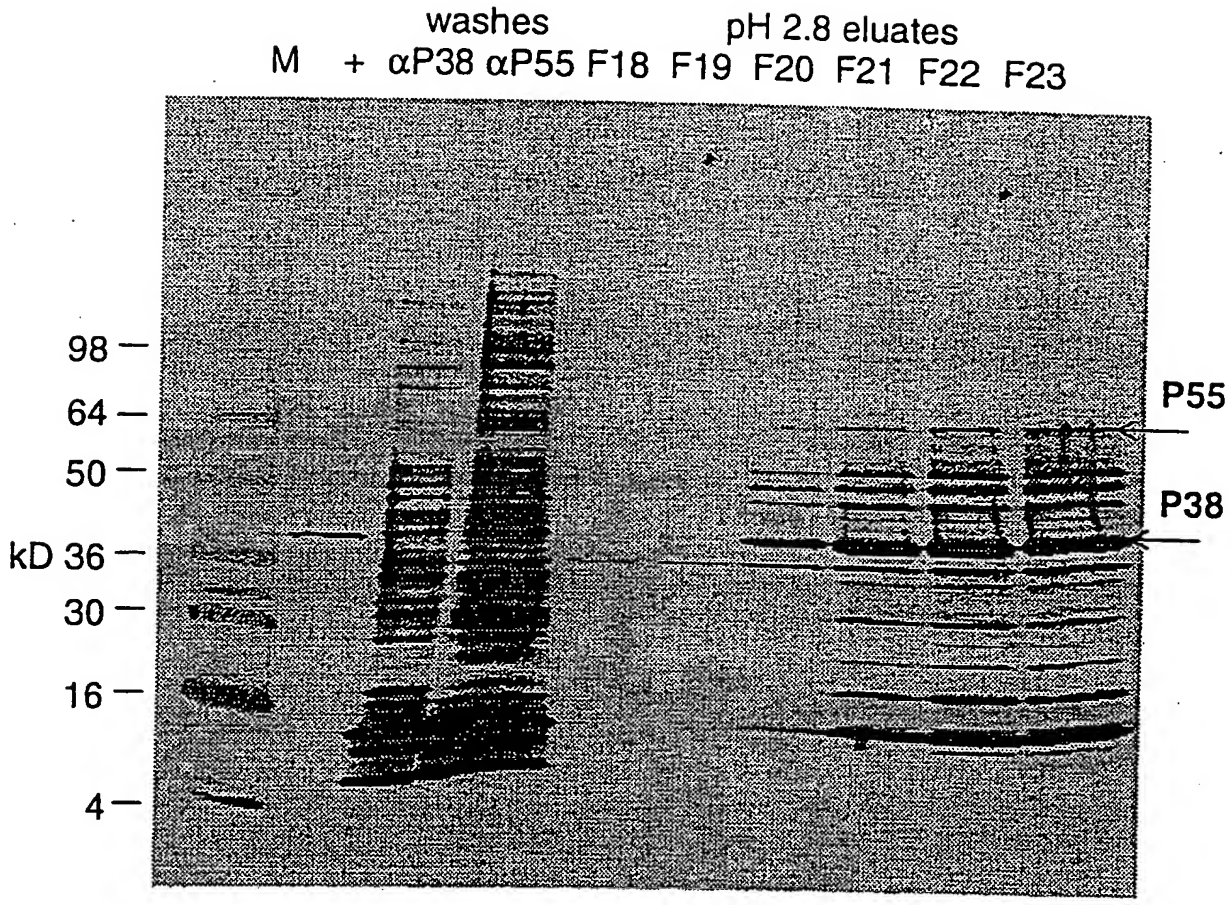

washes
pH 2.8 eluates
M + αP38 αP55 F18 F19 F20 F21 F22 F23
98
64
50
kD 36
30
16
4
P55
P38

Figure 13

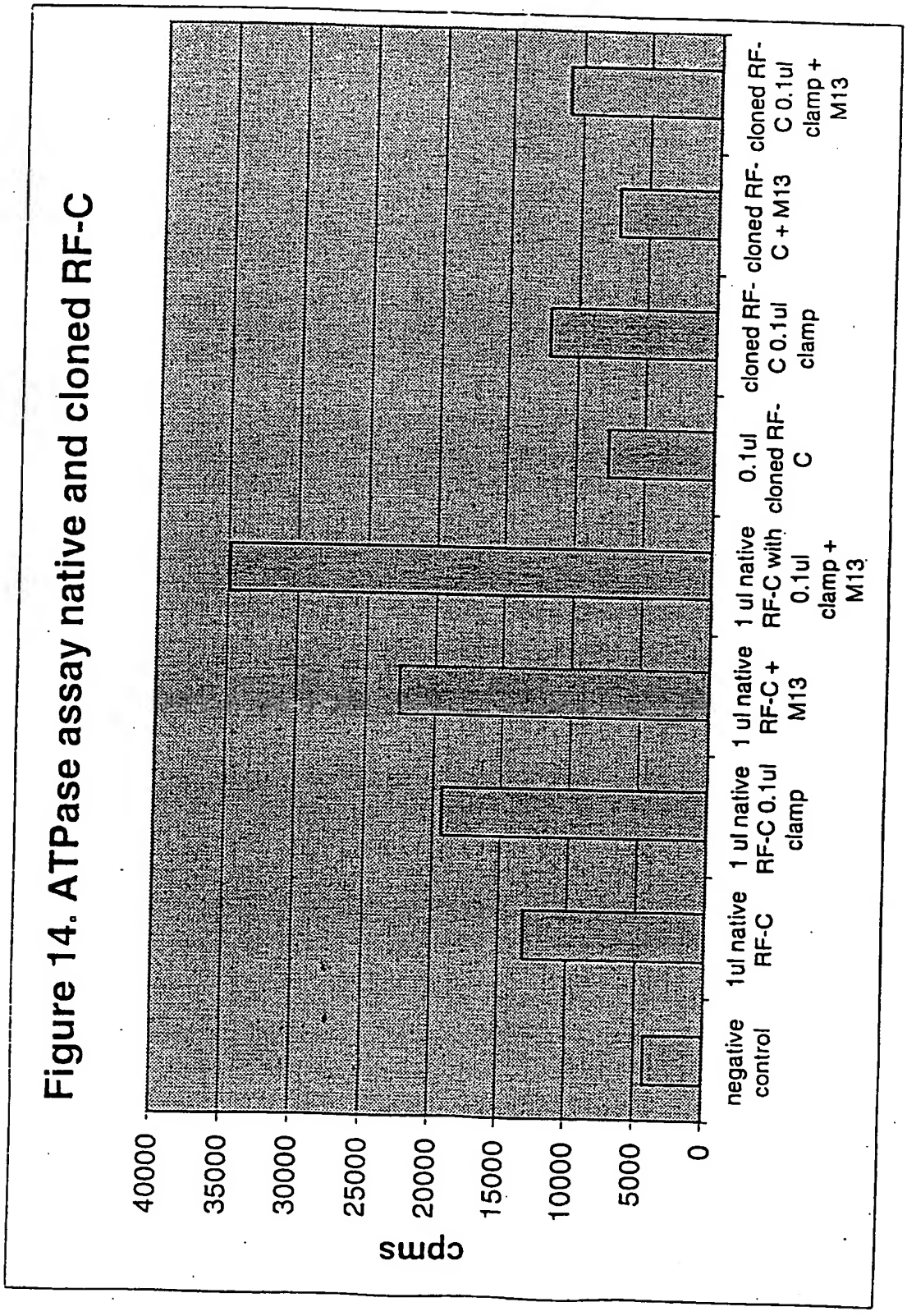
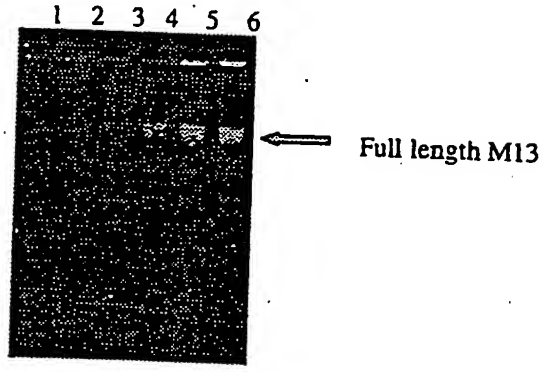


Figure 14. ATPase assay native and cloned RF-C
cpms
40000
35000
30000
25000
20000
15000
10000
5000
0
negative control
1ul native RF-C
1 ul native RF-C 0.1ul clamp
1 ul native RF-C + M13
1 ul native RF-C with 0.1ul clamp + M13
0.1ul cloned RF-C
cloned RF-C 0.1ul clamp
cloned RF-C + M13
cloned RF-C 0.1ul clamp + M13

Native clamp loader stimulation of cPfu/clamp primer extension on M13



Volume of native clamp loader

1. 0
2. 0.01 ul
3. 0.1 ul
4. 0.5 ul
5. 1 ul
6. 2 ul

Figure 15

FIGURE 16

ATGAGT$_7$GCATTTACAAAAGAAGAAATAATCAAGAGGATCCTGGAAGAAGTGGAAGG
AATAACTCTAGAAGAAATTGAGAACCAAATAAGGCAAATAATGAGGGAAAACAATAT
TTCAGAGCATGCAGCTGCTCTCTTACTAGCAGAAAGGCTGGGAGTTGAAGTTACCA
AAAGAGAAGAACAACCTTTAATGAAGATTAGCGACCTATATCCAGGAATGGATCCC
CACGAGGTCAACATTGTTGGAAGAATACTTAAGAAGTATCCACCGCGAGAATACAC
AAAGAAGGATGGAAGCATTGGAAGGGTTGCCAGTCTAGTTATATACGATGATACTG
GGAGAGCGAGGGTTGTTCTTTGGGATTCAAAAGTTTTGGAGTATTACAGCAAGCTA
GAAGTAGGGGATGTTATTAAGGTTTTAGACGCCCAGGTTAGGGAGAGCTTATCTGG
TTTGCCTGAATTGCACATTAACTTCAGGGCTAGAATAATTAAAAACCCAGATGATCC
TAGGGTTCAGGATATCCCACCTCTTGAAGAAGTTAGAGTGGCAACTTATACGAGAA
AGAAGATCAGTGAGGTCGAGCCTGGGGATAGATTTGTAGAGCTTAGGGGAACAATT
GCCAAAGTTTACAGAGTTTTGGTATATGATGCATGTCCAGAGTGTAAGAAGAAGGTT
GACTATGACCCAGGAATGGACGTTTGGATATGTCCAGAACATGGAGAGGTTGAGCC
AATAAAAATCACTATTCTTGACTTTGGGCTTGATGATGGCTCGGGATACATTAGGAT
TACCCTCTTTGGAGACGATGCTGAAGAGTTGCTGGGAGTAGGGCCAGAAGAGATT
GCCCAAAAGCTTAAGGAAATGGAGAGCATGGGCATGACTCTCAAGGAGGCAGCGA
GAAAATTGGCGGAGGAAGAGTTCTACAATATAATAGGGAAAGAAATAATCGTGAGG
GGAAATGTAATTGAGGACAGGTTCTTGGGCCTAATCTTAAGGGCCTCCTCCTGGGA
AGAAGTTGACTACAAGAGAGAAATTGAGAGAATTAAGAGGGAATTGGAAGAATTGG
GGGTGATGTGA (SEQ ID NO:)

FIGURE 17

MI$_3$MSAFTKEEIIKRILEEVEGITLEEIENQIRQIMRENNISEHAAALLLAERLGVEVTKREE
QPLMKISDLYPGMDPHEVNIVGRILKKYPPREYTKKDGSIGRVASLVIYDDTGRARVVL
WDSKVLEYYSKLEVGDVIKVLDAQVRESLSGLPELHINFRARIIKNPDDPRVQDIPPLEE
VRVATYTRKKISEVEPGDRFVELRGTIAKVYRVLVYDACPECKKKVDYDPGMDVWICPE
HGEVEPIKITILDFGLDDGSGYIRITLFGDDAEELLGVGPEEIAQKLKEMESMGMTLKEAA
RKLAEEEFYNIIGKEIIVRGNVIEDRFLGLILRASSWEEVDYKREIERIKRELEELGVM
(SEQ ID NO:)

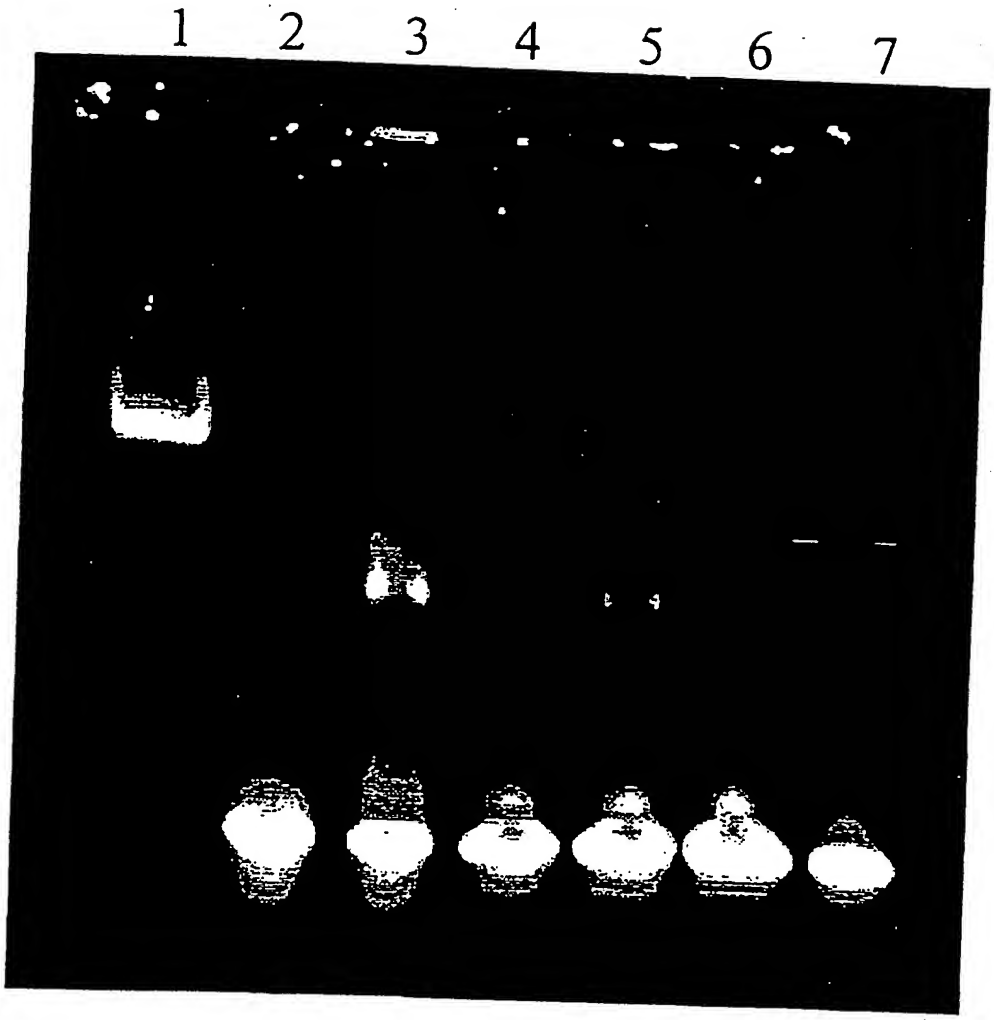
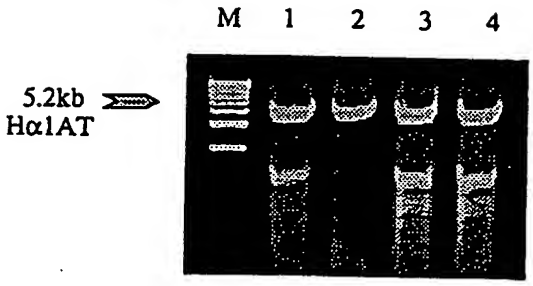


Figure 18 RFA Gel Shift

Pfu Turbo with RFA

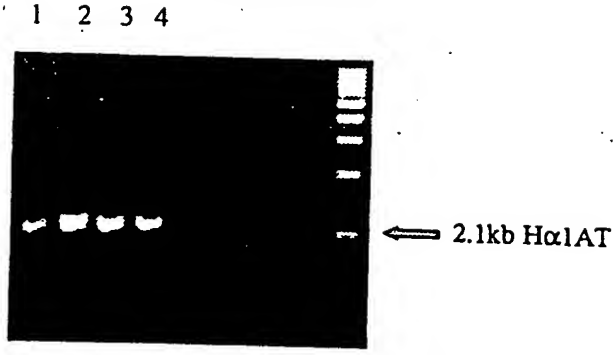


RFA/50µl PCR RXN

1. 0
2. 1µl
3. 1µl 1/10
4. 1µl 1/100

Figure 19

Pfu Turbo with RFA

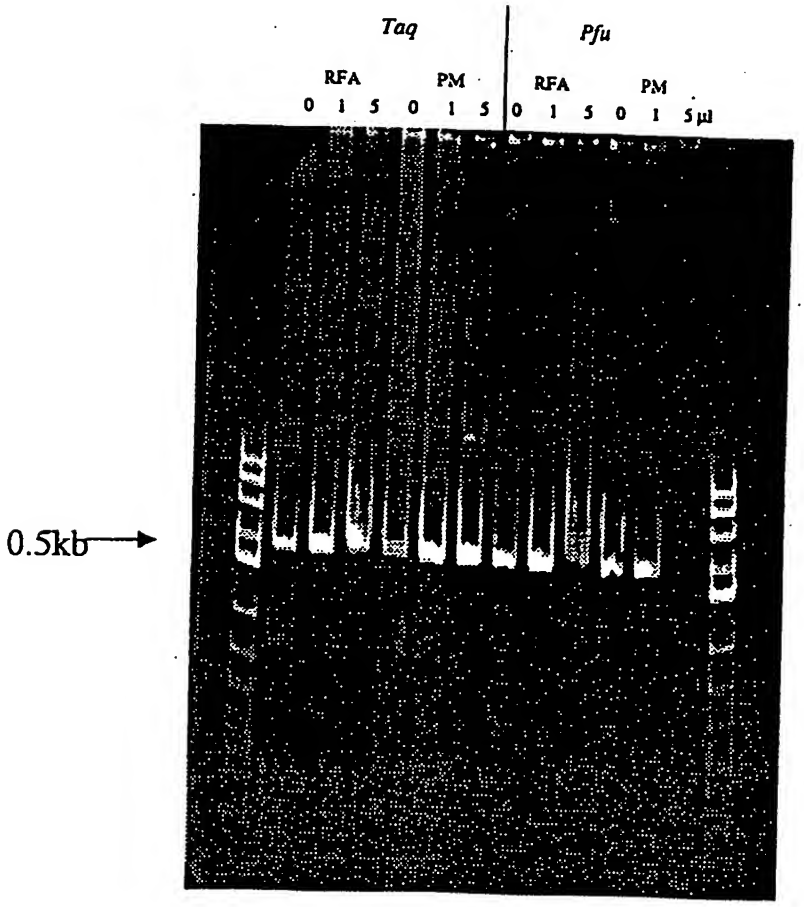


RFA/50µl PCR RXN

1. 0λ
2. 1λ
3. 1λ of 1/10
4. 1λ of 1/100

Figure 20

Effect of RFA and E. coli ssb (Perfect Match) on PCRs using Taq and Pfu DNA Polymerases



RFA= *P. furiosus* RFA
PM= Stratagene's *E.coli* ssb

Figure 21

FIGURE 22

ATGATTGAGGAGCTGTTCAAGGGATTAGAGAGTGAAATCGTTGGACTTCACGAGAT
TCCCCCAAAGAGGGGAGAGTATGGGGAGTTCAAATTCAGGAATGAAGAAGTTAATG
AGTTAGTTAAGAGGCTCGGATTTAGACTTTATTCTCACCAAGTTAAAGCCCTAGAAA
AGCTGTATTCAGGGAAAAACGTAGTTGTTTCAACGCCCACAGCTAGTGGGAAAAGC
GAGATATTTAGGTTGTTTATCTTTGACGAAATACTGTCAAGCCCGTCCTCAACTTTTC
TCTTAATCTACCCAACAAGAGCCTTAATAAACAACCAAATGGAAAAATTCGAAAAAG
AAAACACTATCTTTGAGGAGATTTGTGGAAAAAGAGTTCGAGCAGAAGTCTTAACTG
GAGATACGGAATGGGAAAAGAGAAGAGAAATCATTAGGAGCAAACCAAACGTAATC
TTCACGACACCCGATATGCTTCATCATCACATTCTTCCCAGGTGGAGGGATTATTTC
TGGCTTTTAAAGGGGCTTAGACTTCTTGTCGTGGACGAATTGCACGTTTATAGGGG
GATCTTTGGAACAAATGTTGCTTATGTTTTCAAGAGACTCTTTCTCAGGCTTAAGAG
ATTAAGTTCAAGCCCCCAAATACTTGCCCTTTCAGCAACTTTGAGAAACCCCAAAGA
ATTTGCTGAACAATTTTTTGAGACTGAATTTGAGGAGGTCAAGGAAGCTGGAAGTCC
AAGCCCGAGAAGAATTATAGTCATGTTTGAGCCAAGAAGGTTTACTGGAGAACAAC
TAATCAAGCAAATTGTTGAGAGACTAACTAGAAAGAACATAAAGACCTTGGTATTTTT
TGACTCCAGAAAGGGGACAGAAAGAATCATGAGGCTTTTCCTGTTCTCAGATGCTT
TTGATAGGATCACAACATACAAAGGGACGCTAACTAAGAGGGAAAGGTTTCTAATA
GAGAGAGACTTTAGGGAGGGCAACCTCACAGTTCTCCTAACGACAAATGCACTCGA
GTTGGGAATTGACATTGGAGATTTAGATGCAGTAATAAACTATGGGATTCCTTCAGA
TGGATTGTTTTCACTAATTCAAAGATTTGGTAGGGCCGGAAGGGATCCAAATAGAAT
TGCAATAAACGGGATAATTTTGAGAAGAAATGGATTGGACTACTATTACAAAGAACA
TTTCGATGAGCTCGTTGAGGGAATAGAAAAGGGCCTAGTGGAGAAAATCCCCGTTA
ACTTGGACAATGAAAAGATAGCGAAAAAGCACCTCCACTATGCCATAGCTGAACTT
GGAGTTGTCTCAATTAAAGAAATTGAGGGGAGATGGAAGAGATTCATAAAGACCCT
CGTAGAGGAGGGATACGTGGAAGTTACAAGAAATCCAATAACTGGAGAGGAAGAAA
TAAGACTCAGAAGACCTCCTGTCTATTCTTCAATTAGAACGGCGAGCGATGAAAGC
TACTTCTTAGTCGTGGATGAACCCTGGATAAGGGGAGCTTTGCAGAGGAAGAGGG
GAGCCGAACTTCTCCGTTTTGTAAACTACCTCAAAGTTAGAGGAATGGTAGTTGAG
GAAGTTGATGAGATAGAATTCCACAGAAGTCTACTCCCTGGAATGGTCTACCTTTCA
AGGGGAAGGCCCTACATGGCAGTTGATAAGATAAAGATTGAGAAGTTCCACTTCGT
TTTTGCGAGGCCTCTTCCAATCGAAGAAGAAATAGATACTAGTTCAAGTAAAATTGA
AAACATTGAGATACTTGAGGTTAAAGACGAGAAAACTGTTGGCCCAATAAAAGTGAA
GTTCGGAAGACTTAGAGTAAGGCACGAATACACTGGATACGCCGTGAGGGGAAGA
GACGTTGAAAGGCACGTTAAGAGATTAGAAGAGCTAAAAGATGAGGGGATACTAAG
GGGAGAGATTGACATCGTCCCATACATTTGGGAATCCTGGAAGTTTGCGAGGGTAC
TCTTTGACACCCCCTACATTAGAGAGTTTGAAACTGAAGGTTTCTGGCTTGAGTTTC
CAAACGATATTAGGATAGTTCCCGAAGAGGAGTTTAGGGAATTCTTTGCAGTGGCC
TCTGAGATAGATCCAGAGCTCGCGATGTTCCTCTACAACAGAATTAGTAGAAAATCT
CTATTCCCCACGCTTCTGGGAGCAACCACACACTACATAAGGAGTTTCATCCTTCAC
CACGCCAAAGATAAGGGAGAAGAATTCGCATTTGCCGTAAAAAAGATGATCGACAG
CAAGGATGGGATAGGCTCAGGGCTTCATGCAATTGAGCCCAATATAATAAAGCTTG
CTCCAGTTGTGACTCATGTGGATTCGAGAGAAATAGGCGGCTACAGCTACGATGAC
TTCCATGGAAAGCCAGTGATCTTCATCTATGATGGGAATGAAGGCGGAAGCGGAAT
AATTAGGCAGGTGTATGAGAACGTAGAAAAGCTGATGTACAGGAGTTTGGAGCATA
TAAAGAAGTGTCCATGCAAAGACGGCTGTCCTGCCTGCATATATTCTCCCAAGTGC
GGAACTTTCAATGAATTCCTCGACAAGTGGATGGCAATAAGAATATGGGAAAAAGT
CCTTCCTTAA (SEQ ID NO:)

Figure 23

ATGTTAATAGTTGTAAGACCAGGAAGAAAAAAGAATGAGCTCGAGGCTTTTATAATTGAAAACCCTCCAGAA
AAGCTCTCTCAAAGAAGAAATTTAAAAGCTGATAGGGTAGTTAGGCTCATAATGAGAGATAATAGACTTTTT
AAAGCTCTTGAAGGAAGTCAGTATTTAAATCCAAAGGAAGTGGAGAGAGCCCTTAGAAATTCAAGGATAGTT
CTGGTGAATGCCAACGAGTGGGAAGAGTACTTTAAGAAGAGGTTAATGAACAAAAGAGTTGAAAAAGCTGAC
ATCTGTAGGCTCTGCCTTCTCAATGGGAAGATTACAGTACTCACTGAGGGAAACAGGATAAGATACAGAGAT
GAATACATATGTGAAAGTTGTGCCGAGGAGGAGTTGAAGAGAGAGTTAAGATTTCGATTTAATTCCATAGGA
ATGCTTGAACAGGCAAAGAAGCTTTTAGAGAGATTCAGAGATTTAGACAAGGTGATTTCAATTTTTGATCCA
TCCTTTGACCCCACTAAGCATCCAGAGATAACAAAATGGGATGAGCTAAAGGCCAAGCATATAAGGGTCGAG
AAGATGCATATAGATGAGCTCAACATCCCCGAAGAATTCAAAAAAGTTCTAAAGGCCGAAGGAATAAACGAA
CTACTCCCCGTTCAGGTGCTAGCGATTAAAAACGGCCTCCTAGAGGGGGAGAATTTATTGGTGGTTTCAGCA
ACTGCGAGTGGAAAAACTCTAATCGGAGAGCTTGCAGGTATTCCTAAGGCTCTAAAGGGAAAGAAAATGCTG
TTCCTAGTTCCTCTAGTAGCTTTAGCAAACCAAAAGTACGAGGACTTCAAGAGAAGATACTCAAAGCTTGGA
TTAAAAGTAGCCATTAGAGTCGGAATGAGCAGGATAAAGACCAAGGAAGAGCCAATAGTTCTGGATACTGGA
ACAGATGCACACATAATAGTGGGGACTTACGAAGGAATAGACTACCTTCTCAGAGCTGGTAAAAAGATAGGA
AACGTTGGAACGGTTGTAATAGATGAAATACACATGCTCGATGATGAGGAGAGAGGAGCTAGGCTAGATGGG
CTCATTGCAAGGTTAAGGAAGCTCTATTCAAATGCCCAATTTATTGGGCTTTCAGCAACCGTAGGAAACCCT
CAGGAGTTAGCCAGGAAGCTAGGGATGAAACTAGTGCTTTACGATGAAAGGCCCGTTGACTTAGAGAGGCAT
TTAATAATTGCGAGAAATGAGAGTGAGAAGTGGAGGTATATAGCTAAGCTGTGCAAAGCCGAGGCCATGAGA
AAGAGCGAGAAGGGATTCAAGGGGCAGACGATAGTATTTACATTTTCAAGGAGAAGATGCCATGAGCTTGCC
TCATTCCTAACGGGGCAGGGATTGAAGGCTAAGGCCTACCACTCGGGCCTCCCCTATGTTCAGAGAAAGCTT
ACCGAAATGGAGTTTCAAGCTCAAATGATTGATGTAGTTGTAACAACAGCTGCTTTAGGAGCGGGAGTTGAT
TTTCCAGCATCCCAAGTCATCTTCGAAAGCTTGGCCATGGGAAACAAGTGGATAACAGTTAGGGAGTTTCAC
CAAATGCTTGGCAGGGCTGGAAGGCCACAGTACCATGAGAAAGGTAAAGTTTACATAATAGTCGAGCCTGGG
AAAAAGTACTCAGCTCAGATGGAGGGAACTGAAGATGAAGTCGCCCTCAAGCTCTTGACTTCACCCATAGAA
CCAGTAATTGTTGAGTGGAGCGATGAATTTGAAGAGGATAATGTCTTAGCTCATGCCTGTGTGTTTAATAGA
CTTAAAGTTATTGAAGAAGTTCAATCCCTCTGCCTGGGAGCAAACCAAAGTGCTAAAAATGTTTTGGAAAAA
CTTATGGAAAAGGGGCTCGTCAAAATATATGGAGATAAAGTTGAAGCAACCCCATATGGAAGGGCGGTGAGC
ATGAGTTTCCTACTTCCTAGGGAGGCAGAGTTCATCAGAGATAACTTGGAGAGCACTGATCCAATTGAGATA
GCAATTAAACTGCTACCGTTCGAAAACGTTTACCTCCCAGGATCGCTCCAGAGGGAAATAGAGTCAGCTGTT
AGAGGAAAGATAAGCTCAAACATCTTTTCAAGCTCCTTTGCATCAGTGCTAGAAGAGCTTGACAAGATTATA
CCCGAAATAAGCCCAAATGCTGCAGAAAGGCTATTCCTAATATACCAAGATTTCTTCAACTGCCCAGAGCAA
GACTGTACGGAGTTTGCAATGGAGAGAATTGGGAGAAAGATCATTGACTTAAGAAGAGAGGGATACGAGCCC
TCAAAAATCTCTGAGCACTTTAGGAAGGTCTATGCATTAATATTATACCCTGGAGATGTTTTTACATGGTTA
GACGGAATTGTGAGAAAACTCGAGGCAATTGAAAGAATAGCCCGAGTGTTCAATAAGAGAAGAGTGGTAGAA
GACACAATCAGGGTTAGAAGGGAAATTGAAGAAGGAAAAATTTTGAAGGGTGAGAGACGATGA

FIGURE 24

ATGCACAAATACTTCTTTCCATTACCTGCAACTAAGTCAACTTTCTTGCTCCCTGCC
GACCTCACCACAGCAAATCCATGCTTTTCCAAGAGCTTAATCAATTCTCTCTCTGCC
TGGGCCCCTTTTCTATACATACAATGTTTTTCCTATCTACCTCTTATAAACTTTTTAAA
CTCCTTGACATACCCTCTCGAGATGCACATATTGATAAAAAAGGCAATAAAAGAGAG
ATTTGGAAAGTTGAATGCCCTTCAACAATTAGCCTTTCATAAAATTAGGGGAGAAGG
TAAAAGTGTTTTAATAATAGCTCCGACAGGAAGCGGAAAAACTGAAGCCGCAGTAA
TTCCAATCTTAGACGCAATACTACGGGAGAATCTTAAACCTATAGCAGCTATTTATAT
AGCCCCATTGAAGGCACTAAATAGGGACTTGCTAGAGAGACTAAAGTGGTGGGAA
GAAAAAACTGGGGTAATAATAGAGGTTAGGCATGGGGACACGCCTACCTCAAAAAG
ATTGAAGCAGGTAAAAAATCCTCCCCACCTATTAATTACAACCCCTGAAATGCTCCC
TGCTATTCTTACGACAAAGTCCTTCCGTCCCTATCTTAAGAACACTAAATTTATCGTG
ATAGACGAGATTGGTGAACTTATAGAGAATAAAAGAGGAACCCAGCTAATCCTAAAT
CTAAAAAGACTTGAATTAATTACAGAAGATAAACCAATAAGGATTGGCCTTTCTGCA
ACAATTGGAAGTGAAGAAAAGGTAAGGCTTTGGATGGAAGCGGATGAAGTGGTAAA
GCCTCGACTAAAAAAGAAGTACAAATTTACCGTTTTATACCCTCAGCCAATTCCAGA
GGATGAAAAGCTTGCTGAAGAGCTCAAAGTTCCAATAGAAGTTGCAACGAGGCTAA
GAGTTGTGTGGGATATTGTAGAAAAGCACAAGAAGGTATTGATCTTTGTTAATACCC
GACAATTTGCAGAGATCTTAGGGCATAGACTTAAAGCTTGGGGAAAACCTGTTGAA
GTTCACCATGGTAGCCTTTCAAGGGAAGCAAGAATAGAGGCAGAGAAGAAACTTAA
GGAAGGAAAAATAAAAGCACTAATTTGTACCTCATCAATGGAACTTGGCATTGACAT
AGGGGATGTTGATGCAGTTATTCAGTACATGAGTCCTCGACAGGTAAATAGGCTAG
TCCAGAGAGCTGGAAGAAGCAAACATAGACTGTGGGAAACAAGCGAGGCTTACAT
CATAACCACAAACGTAGAAGATTATCTCCAAAGCTTGGCAATAGCAAAGCTCGCACT
AGAAGGAAAACTGGAAGATGTAAATCCCTACGAAAATGCCCTTGATGTCCTGGCTC
ACTTTATAGTTGGTTTGACAATAGAATACAGAAATGTTAACATTACTGAACCCTATTC
CCTTGCGAAATCTACTTATCCCTACAGAAAGCTCTCCTGGGAAGACTATCAGAAAGT
TTTAGAGATTTTAGAAGAGGCTAGAATAATAAGAAGAGATGGAGATGCAATTAAGCT
GGGAAAAAATGCCTTTAAGTATTATTTCGAGAACCTCTCAACAATACCTGACGAAAT
AAGTTATGCAGTTATAGATATTGCAAGTGGAAAATCTGTTGGAAGACTAGATGAAAA
CTTTGTTACGGAACTTGAAGAGAGTATGGAATTCATCATGCATGGAAGAAGCTGGA
TCGTGCTGGAAATTAACGAAAAAGAAAGGATAATAAAGGTTAAGGAGAGCAACAATT
TAGAAAGTGCACTGCCAAGTTGGGAAGGGGAGCTCATTCCAGTTCCTTTGGAAGTT
GCAGAATTTGTTGGAAAGCTGAAGAGAGAGCTCCTATGGGACAAAGAGAGAGCATT
AAAACTGCTTGAGGGCGTTGAATTTAATAAGGAAGAACTCGAGGTTGCAATTTCCCA
ACTAGTAGAATCAGAACCAGTGGCGAGTGATAGAGATATCATTATAGAATCCTATCC
AAAATTTGTGATAATTCATGCTGATTTTGGAAATAAAATTAACGAAGGGCTCACAAG
ATTTATCTCAGTGTTTTTATCCGCCCGATATGGGAATATTTTCCTCCCAAGAAGTCAA
GCTCATGGAATTATAATTAGAAGCCCATTTAGGCTTAATCCTGAAGAAATAAAGGAA
ATACTGTTAATGAAAGCAGAAGTTGGAGATATTGTTGCTAGAGGAATTAGAGACACT
CCAATATACCGCTGGAAGATGAGTGCAATTGCTAAGAGATTCGGTGCCCTAAGAAG
GGACGCGAGAATAAAAAAAGTAGAAAGGCTGTTTGAAGGGACAATAATAGAGAAGG
AGACTTTTAATGAAATTTACCATGATAAAATCGACATTGATAAAACAGAGAAAATTCT
AGAAAAAATAAGAAAGGGAGAAATTAGAATGAAAACTTTGTTCAGAGAGGAAATAAC
GCCTCTTTCCTCTTCTTTGGCAACCCTAGGAGGAGAGTTTCTAATTAGAGATATACT
TACCCAGGAGGAAGTAGAAGAGATATTTAGGGAGAAGTTACTCGATGCTGAGTTAG
TCATGGTTTGTACAAACTGCGGATTTTCCTGGAGAACAAAAGTTCGCAGGGTTATG
GATAGAGTCAATGAGTTAAGCTGTCCCAAGTGTGATTCCAAAATGATAGCTCCTCTA
CACCCCAAAGATTCCGAAACTTTCATCTCAGCTCTCAAAAAGTTAAAAAGAGGAGAA
AAGCTTAGTAGGGAAGAAGAAAAGTATTACCTTAGAGGTTTAAAGGCGGCTGATTTA
CTTAAAGCCTACGGGAAGGACGCTCTTTTAGCATTAGCTACCTATGGGGTTGGGGT

FIGURE 24 cont.

AGAAAGCGCCACCAGAATACTTAGGGATTATAGAGGAAAATCCCTTATAAAAGCACT
TATCGAGGCAGAGAAACACTACATCCAAACTAGAAAGTTTTGGGAATAG (SEQ ID
NO:)

FIGURE 25

GTGATGTTATTAAGGAGAGACTTAATACAGCCTAGGATATATCAAGAGGTAATATAC
GCCAAGTGCAAAGAAACAAACTGCTTGATTGTTCTGCCCACAGGATTAGGTAAGAC
GCTGATAGCTATGATGATAGCAGAGTATAGATTAACGAAATATGGCGGAAAAGTTCT
AATGCTCGCCCCCACTAAGCCTCTCGTTCTTCAACATGCGGAAAGTTTTAGGAGGC
TATTTAACCTCCCTCCAGAAAAAATTGTAGCACTTACTGGAGAGAAGAGCCCAGAA
GAGAGAAGTAAGGCCTGGGCGAGAGCAAAAGTAATTGTAGCCACTCCTCAAACTAT
TGAAAATGACTTATTGGCGGGAAGAATATCTTTAGAAGACGTTTCGCTAATAGTATT
CGATGAAGCTCACAGAGCTGTGGGCAATTACGCTTACGTCTTTATAGCAAGAGAGT
ATAAAAGACAGGCCAAAAACCCACTTGTTATAGGGTTAACAGCCTCCCCTGGGAGC
ACTCCTGAAAAGATCATGGAGGTAATAAATAACTTGGGAATTGAGCATATTGAATAC
CGCTCCGAAAATTCTCCCGATGTTAGACCTTACGTTAAGGGAATAAGGTTTGAATG
GGTTAGGGTTGATCTCCCAGAAATATACAAGGAAGTAAGGAAACTTTTAAGAGAAAT
GCTTAGAGATGCCCTTAAACCGTTGGCAGAAACTGGACTTCTTGAATCTTCTTCCCC
AGACATTCCAAAGAAAGAAGTTCTTAGAGCTGGGCAAATAATAAACGAAGAAATGG
CGAAAGGTAATCATGATCTCAGAGGCTTGCTTCTCTATCACGCAATGGCTCTTAAGC
TACATCATGCAATTGAGCTGTTGGAAACCCAAGGGTTATCCGCCCTGAGGGCTTAT
ATAAAGAAGTTGTATGAGGAGGCAAAAGCGGGATCAACAAAGGCTAGCAAGGAAAT
ATTCTCGGATAAGAGAATGAAAAAGGCAATCTCACTTTTAGTTCAAGCGAAGGAGAT
TGGGCTTGATCACCCCAAGATGGACAAGTTAAAAGAAATAATTAGGGAACAACTCC
AAAGGAAACAAAATTCCAAAATCATAGTTTTCACTAACTACAGAGAAACTGCAAAAA
AGATAGTCAATGAACTTGTGAAAGATGGAATAAAAGCTAAAAGGTTCGTTGGACAG
GCCAGCAAAGAAAATGACCGTGGACTGAGTCAGAGAGAGCAGAAATTAATTCTTGA
CGAATTCGCTAGAGGAGAATTCAACGTTCTAGTGGCAACGAGTGTAGGAGAGGAA
GGACTTGACGTGCCGGAAGTTGATTTGGTTGTGTTTTATGAGCCAGTACCATCTGC
CATAAGGAGCATCCAAAGAAGGGGTAGAACTGGCAGGCATATGCCGGGGAGAGTT
ATAATCCTAATGGCCAAGGGGACTAGAGATGAAGCATACTACTGGAGTTCCAGGCA
AAAGGAAAAGATAATGCAAGAGACAATAGCTAAGGTGAGTCAGGCAATTAAAAAGC
AGAAGCAAACTTCTCTAGTTGATTTTGTGAGAGAAAAAGAGAGCGAAAAGACCTCTC
TAGACAAGTGGTTGAAAAAGGAAAAAGAAGAAGCAACTGAAAAAGAGGAAAAGAAG
GTAAAGGCTCAAGAGGGTGTAAAAGTCGTCGTAGATAGCAGAGAGCTTAGGAGTG
AGGTTGTGAAGAGACTTAAACTTCTTGGTGTAAAGTTAGAGGTTAAAACGCTCGATG
TGGGAGATTATATAATTAGTGAGGACGTTGCAATTGAGAGGAAGTCAGCTAACGAC
TTCATTCAGTCAATTATTGATGGTAGACTTTTTGATCAAGTTAAGAGGCTCAAAGAG
GCATACTCAAGACCGATAATGATAGTCGAAGGTTCTTTATACGGAATTAGAAACGTC
CATCCAAATGCAATAAGGGGGGCAATAGCAGCGGTAACCGTAGACTTTGGGGTCC
CAATAATATTTTCATCTACTCCAGAGGAAACCGCTCAATACATCTTTCTAATTGCAAA
GAGGGAGCAAGAGGAGAGAGAAAAACCTGTGAGAATTAGAAGTGAGAAGAAGGCC
CTTACCCTTGCCGAGAGGCAGAGGTTAATAGTTGAGGGATTACCTCACGTCTCAGC
AACTCTAGCTAGGAGATTGTTGAAGCACTTTGGAAGTGTGGAAAGGGTATTCACTG
CAAGCGTTGCTGAGTTAATGAAAGTTGAAGGCATAGGAGAGAAGATTGCTAAGGAG
ATTAGAAGGGTAATAACTGCCCCATATATAGAGGATGAGGAGTAG (SEQ ID NO:)

FIGURE 26

TTGAAAGGGTTGTTTAGGGACGTTATCCTCCACAACCCCCACCTTTTTGTTTATTCC
TATTCTGATAAAGGCATCATTCCTTTCAAGCATCAGTTCCAGACCCTCTATCATGCC
ATGCTCATGAGGCCAGTGAGGCTAATGATAGCTGATGAGATAGGTCTCGGAAAGAC
CATTCAAGCTCTTTTAATAGCCAAGTACCTCGATTTTAGGGGAGAGATTGAGAAAGC
CTTGATAGTCGTTCCAAAAGTTCTGAGGGAGCAGTGGAGGGAAGAAGTAAAGAGG
ATCTTAGAGGAAGCTCCGGAAGTGATAGAGAATGGTAGCGAAATTGAATGGAAGTT
GAAAAGGCCGAGGAAGTACTTCATAATATCAATAGACCTAGCTAAGAGATACACCG
AGGAAATACTCCGTCAAAAGTGGGATTTAGTAATAGTTGACGAAGTCCACAACGCC
ACCCTGGGAACACAGAGATATGAGTTCTTAAAAGAACTAACCAAGAACAAGGATTT
GAACGTTATATTCCTTTCAGCAACCCCCCACAGGGGAAACAATAGAGATTACCTTG
CGAGGCTTAGGCTCCTCGACCCAACTATACCAGAGGAAATATCCCCAATGCACGAA
AGGAAGATCTACATGAAGTCAAGAGGGACATTGGTACTAAGGCGAACTAAGAAGGT
TGTCAACGAACTTGAAGGAGAAGTGTTCAAGAAGTGTCACTTTGGGGCTGTCGTGG
TAGAAGTTAGCAGAGAGGAGAGGGAGTTCTTTGAAGAGTTAAATAGAGCGCTATTC
GAGCTGATTAAGGATCAAGCTGATTACTCTCCCTTAACTCTTCTTGCAGTAATCATT
AGGAAGAGAGCCTCGTCCAGCTACGAAGCGGCTCTAAAAACCCTAACCAGGATCG
TTGAAAGCGCTTATATAAGTGGGCAAGAAAGAGCCAGAGGCGTTGAATCATACATT
GAAAAGATCTTTAGAATGGGGTATGAGGAATTGGAAATAGAAGAATTTAACGAGATA
GATGATGCGATACACAAAATAATAGATGAATATAGGGGATTCTTAACTGAAGAGCAA
CTCGAAAGGCTTAGAAGAGTTCTCGAGCTTGGAAAGAAAATTGGCAGCAAGGATAG
CAAGCTTGAGGTTATATCCGATATAGTTGCTTATCACATTAGGAACGGCGAAAAGGT
CATAATATTCACGGAATTTAGAGATACCCTCGAATACGTACTTGAGAGGTTACCAGA
TATCCTAAGGAGAAAGCACGGCATTGTTTTGGAAAAAGATGACATTGCAAAACTTCA
TGGGGGCATGAAATCTGAGGAAATAGAGAGGGAAATCAACAAGTTTCATGAAAGGG
CTAACCTATTAGTCTCTACGGATGTTGCATCCGAAGGACTTAACCTGCACGTTGCAA
GTGTTGTAATAAACTACGAGGCCCCCTGGAGCCCAATAAAGCTCGAACAGAGGGT
GGGAAGAATATGGAGGCTCAACCAAACGAGAGAAACCAAAGCATATACCATATTTC
TTGCAACGGAAACGGACTTGGATGTTCTAAACAACCTCTATAGAAAGATTATGAACA
TAAAGGAAGCCGTGGGAAGTGGACCCATTATTGGAAGGCCAATATTTGAAGGAGAC
TTTGAAAATCTATGGAATGAAGGTGCCGAGGAAGAAAATAGAGAAGTCTCAGAGTA
TGAGCTTATCCTAGCCTCAATTAAGGGAGAACTCAAGGGCTATGCCGGGGCTCTAG
TTAGGACTCTCAGAATCCTAAAGCAGAAAGTGGAGGGAGCAGTTCCTGTAAATCCT
GCGGGAAGCATAAGGAGAGAGCTCGAGATAATTTTAGAGGACACTCCTGATGTGG
AAGTATTAAAGAAAATCGTTAATAGGAACGTTCCAAATCCGTTCCGCTTGGTGAGAG
GACTTTTAAGAGAAGCCGAGGGGATTGAGGGAATTAGAGTATTAGTTAAGGGCTAT
GATGGCTCTATGGATGTGTACTATGCCATATTCTACGACGAAGATGGGAGAGAAAT
TTATAGATATCCAATTCTTGCTGAGAACGGAAAGTACCTTGTTGGATTCAACTTACT
CAAGAGGATTAGTGAGGTACTATCCAAAGAGTACAAGGTCGTTAGAGGGGCAAGTG
AAGAGGTGGACTATAAAGTTAAGACGCTAGTTATGGACAACATATACAATTTAATCG
TGAAGAAGTATCTGGAATACGATAGCTTAAACATCAAAGAAGGTAAAATCTTCAAGA
GGCTTAAGGTTGAAATAAAGAAAGCCCTCGAGGTAAAGGGGATAAGTGAAGAAGAA
TTCGAAGTCATCAAGAGAGTTCCCCCTGAGATTATGGAAGTTCTAGGGTTAGATTCC
ACAAAAATAGAACTACCTACCAACGAATACCTCAAGATCTTCGAAAGGAACTTTGTT
CCTCTGGATAAAATCCTTGAGAGTGAAAAGAAGGCCATGGAAATAGTCATGGAGCT
AGAGAAGAGCAGAGGATATAACGTTGAGGACGTATCTTTAAGGGAGCACTATGACA
TAAGGGCCTTTACAGATGGTGAAGAGAAGTACATAGAGGTCAAAGGCCACTATCCA
ATGCTCCTACTTGCGGAGTTAACGGAAAAGGAATTTGAGTTCGCACAAAAAAATGAA
GATAAGTACTGGATATACATAGTCTCGAACATTGCCAAAGACCCCGTAATTGTAAAA
ATTTACAAACCATTTTCCCAGGATAGAAGAGTATTCGTGGTTAAGAATGGGGAAGAT

FIGURE 26 cont.

GTTGAGGTTAATATCAACATTGAGATAAAGAAGAAAGATAGGCATTTACTTAAGTTA
AGCTAG (SEQ ID NO:)

FIGURE 27

GTGATTACTTTGGAGCTACATCCAAGTGAGATAGCTAGATATTTCGAGCTTGAAGAG
TGTTCCCACTATTTCTCTAACCTACTTTTAAGAAAGAGAGGCGAATTGCAGGAATTT
GAGCCGATAATAAGGAGAAAAGAAATAGAAACCATAGAGCTCGCCAAATGGGGAGA
CGAGTTCGAGCTCTCCCTTCTTCAGGAATTTAAAAAAGGTGAAGCATTAAAAAAGCT
TGGAGTTAAAGAACTACCAAGATTCTATGGTTTTTTAACGGAAAACGACACCCCTGT
AAGAAAGTTCTTTGAAAAGTACTTTAAAGATGGAATAATAGTGGAAGAAGATCCAGA
CAAACTTTTAGAAATTATAAACAGTGAGAAAAGTGCCGTTATCTATCAAGCCCCCTT
AAAAGGCAGAATAGGGAAATTTGATGTCTCAGGAAGGGCAGACTTCATAATAAAGG
TTGGGAAAACACTTTACCTACTCGAGGCTAAGTTTACTAAGGAAGAGAAGTTCTACC
ACAGGATTCAGGCCATTATCTATGCTCACCTTCTAAGTCAAATGATCGAAGGTTACG
AAATTAAACTAGCTGTTGTAACAAAGGAGAACTTTCCCATTCCCTCAAACTTCCTAA
GATTCCCAGGAGACGTGGAAGAGTTAAAGATAACCCTAGAAGAAAAGCTTGGTGGA
ATACTAAGAGAACAAGAACTTTGGATAGACGCAAGGTGTACTACTTGCCCCTTTGA
GGCTTTATGCTTGTCTAAGGCTCTTGAGGAAAGAAGTCTAGGACTATTAAGCCTTCC
CCCTGGGATAATTAGAATACTCAAAGAAGAAGGGATAAAAGACTTAAAAGACATGG
CTAAGCTATTTGAATTCAAAGAAAATTCCCCTACAAACTTTGAAGAGCCCTCAATAAA
AGATCCAAAGAAGACTCAAGAGATAGCAAAAAGAACGGGAATAAACTTACTAAAGC
TCTCAAGGATAGCTCAGGCAATCCTTAAATATTTAGATGAGGGAGAAACAACACCC
CTGTTCATCCCCAGGACGGGGTATAATCTGCCAATGGATGAGAGAGTAGGTGATGT
TGAGCCCTCTTACTATCCTCCAAGGAGCTTAGTGAAAGTGTTCTTCTATGTCCAGAC
AAGCCCAATAACAGACACAATAATCGGAATTTCAGCCCTTGTAAAGAATAGGCAAAA
TGGAGAGCGGATAATTGTTAAGTTCGTCGATGAGCCCCCCATAGAAGTTTCAGATG
CCCAAGAAAAGGAGAGAATGCTTCTAATTGAGTTCTTTAGGGATGTTATTGATGCCG
TAAAGTCACTATCTCCAACCGATAAAGTCTACCTACACATGTACTTTTACAATAGAAA
ACAGAGAGATGACCTTATGGATGCCGTAAAGAGACACAAAGAGATAAGAGAAAACA
ATGCAGTCATGGCCTTGCTAAGCTTGAGAAGAGCCATAGATTGGGAGAGCTTTTCA
ATAATAAAGGATGAGATAATAAGGAGGCATGCCTTACCACTTTCTCCTGGCCTGGG
ATTCGTTACAGTTGCTACTCAGTTTGGATACAGATGGAGAAGGAACAAAACCTTTGC
GCGAATGCTTGAGGTTGTAGCAAGAAGAGAAAATGGTAAGATAAATCTCAAAACTCT
CCTTAACATTTCTGAAACGGGAATTGGGCCAGAATATTATCCAATCATCGATAGGGA
TAACGAAGGAATACCCTTCACACTTTTCTGGAGCGCACTGGTCAAATTAGCTACTGA
GGAAGACAATTCAAGAATTAAGAGGGATATAAGGGACATACTCTCCCAAATGGTTG
AGGCCCTCAAAACAATTGAAGAGAGAATTCCCGAGCAATATAAAGACGCCTTCGTG
AAAAAAGAGGGAATACCCAAAGAAGATCTCGAAAACTTTGACATAAAGAAGGAAGA
ATTAGCTGATATCCTTCTTGAATACTTACAATTAGAGTTCGATGCAAGATTTAGAGAA
CGATCCGAATACTATAGGCTTCCCCTATCAATAAGAGCATACTCAGAGGAATCAGC
ACTAATTAAGATAGAAAACATTGAAAAGAAGAAAAATGACTGTCTGTTGTTTGGAAA
AATCGTGCTAATTGACGAAAATGGAAGAATAAAAGAGTATAATCCAAAAGAAGTTCT
TATAGATATTGATGAAGGTTCTCTTGTAGTTGTAACGCCAAAGAAATTCTTAGATAAG
CTAAGAAGAGATCCCGTTCAAAGAATAAGCAAATCACCGTTAGGAATAGTTGAGGC
TATAGATCACGAGACAGGAAAAGTTGTTATAAGGTTAATAAGAGTCTCTCCAGGCAG
ATTTACACTCAAACACTCTAAGTTTAGTTGTAAAAATGGACTATTGACAATAACCTAT
CCTGAAGGGGAAGTGAAAGTTACTCCTGGAGAGATAGTTATAGTAGATCCTAGCGT
CGATGACATAGGAATGGAAAGGGCATACAATGTGCTCTCAGAAATATCCCAAGGGG
AACTCAAGCATGAAATTTATCAGAAGGTCAAAGCAATATACGAAGGGAACACGGAA
TCAAGATACGAAGTCAACATCTGGAAGAAAAAGCACATAGAAGAATTTCTCTCCAGA
GTTAAGAAGATCAACGAAGAACAGAAAAAGTTTGCAATTGACATAAACAACTTTCTA
GTCACCCTTCAAGGCCCCCCTGGGACTGGGAAGACATCAGGGGCCATAGCCCCAG
CAATTCTCGCAAGAGCATATTCAATGGTGAAGGACAAAAAGAATGGCCTCTTTGTAG

FIGURE 27 cont.

TTACTGGAGTCTCACACAGGGCAGTTAATGAGGCCCTGATAAAGACTTTAAAGCTA
AAGAAAGAGCTGGAGAATACATTAAAAGAGCTTAGAAAGATAGATCTAATTAGAGCA
GTCTCTGGGGAAGAGGCAATCAAAATAATTAAAGAGGAACTAGAGAGGGAAATAAA
GGATGATGTCGACAGAATTAGATTTACAGCACAAGAAATTACCCACTCTTCAAAGCA
AAGATCATTAGACAAATATTTTGCTAATTCTGGAACTGTGAGGATAGTATTTGGAAC
ACCACAGACTTTGAACAAGCTTATGAAGAATACAAAAGAAGTCGAACTAGTTGTCAT
AGATGAAGCTAGTATGATGGACTTACCAATGTTCTTCCTCTCAACAAAAGTTTGTAA
AGGTCAAGTTCTCTTGGTCGGGGATCACAGGCAGATGGAGCCAATTCAAGTCCATG
AATGGCAATTAGAGGACAGAAAGACATTTGAAGAGCACTATCCATTCCTTTCAGCCC
TTAACTTCATTAGATTTCTCAGGGGAGAGTTGGATGAAAGAGAACTTAAGAAGTTTA
AGAGAATCCTTGGAAGGGAACCTCCAGAATGGAAGAAGGACAAGAACGAGGTTCT
CCCTCTCTATAGGTTAGTAAGAACTTATAGGTTGCCCCAGGAAATAGCTGATCTACT
GAGTGATGCAATATACAGAGCAGATGGCATAAAATTGATTAGTGAAAAGAAAAAGA
GGAGAAAGATAATTGCCAGGCACAAGGATGAGTTTCTATCGATAGTTTTAGATGACA
GGTATCCTTTCGTTCTAATACTTCATGACGAGGGCAATTCCACAAAGATTAACGAGC
TGGAAGCAAAGATAGTAGAGAAGATAATCAAAAGAGTAGAGAATATTGATATAGGA
GTTGTAGTTCCATATAGAGCTCAAAAGAGATTAATAGCTTCATTAATAGATAGTGCC
CAGGTGGACACAGTTGAGAGATTCCAAGGGGGAGAGAAATCTTTAATAGTAATTTC
AATGACTTCCAGCGACCCCCGCATACCTGGGAAAGGTTTTTGA (SEQ ID NO:)

Figure 28

ATGAACATAAAGAGCTTCATAAACAGGCTTAAGGAGCTAGTTGAAATCGAGAGGGAAGCTGAAATAGAGGCT
ATGAGGTTGGAGATGAAAAGGCTTAGCGGAGTGGAGAGGGAGAGGTTAGGTAGGGCAATTCTCAGCTTAAAC
GGTAAAATCGTTGGTGAAGAGCTCGGTTATTTCTTGGTTAAGTACGGAAGGAATAAGGAGATAAAGACCGAG
ATCAGCGTTGGGGATTTGGTTGTTATAAGCAAGAGGGATCCCCTGAAGAGCGACCTCCTGGGAACTGTTGTT
GAGAAGGGGAAGAGATTCATCGTCGTTGCCTTAGAACCAGTCCCAGAGTGGGCCCTTAGAGATGTGAGGATA
GACCTCTACGCCAACGATATAACATTCAAGAGGTGGATCGAAAACCTCGACAGGGTTAGGAAGGCTGGAAAA
AAGGCTTTAGAGTTTTACTTAGGTTTAGATGAGCCTTCCCAGGGGGAGGAAGTGAGCTTTGAACCCTTTGAT
AAGAGCCTAAACCCCTCTCAAAGGAAAGCGATAGCTAAGGCTTTAGGTAGTGAAGACTTCTTCCTTATCCAC
GGCCCCTTTGGAACTGGAAAGACGAGGACTTTAGTTGAGCTGATTAGGCAGGAGGTAAAGAGGGGGAACAAA
GTTCTAGCTACAGCTGAGAGCAACGTTGCCGTGGACAATTTAGTTGAAAGATTGGCCAAAGATGGAGTTAAG
ATAGTTAGGGTTGGGCACCCAAGTAGGGTTTCGAGGCATTTGCACGAGACAACTTTAGCTTACCTCATTACT
CAGCACGAGCTCTACGGTGAGCTTAGGGAGCTTAGGGTGATAGGGCAGAGTTTGGCAGAGAAGAGGGACACA
TATACAAAGCCGACTCCAAAGTTCAGGAGGGGACTGAGTGATGCTGAGATAATTAAGTTGGCCGAGAAGGGA
AGAGGGGCTAGAGGACTCTCAGCTAGACTAATAAAGGAGATGGCCGAGTGGATAAAGCTAAACAGGCAGGTT
CAGAAGGCCTTTGAAGATGCTAGAAAGCTTGAGGAGAGGATTGCGAGGGATATAATTAGGGAAGCCGATGTG
GTTTTGACAACTAACTCTTCTGCAGCCCTTGATGTTGTTGATGCTACCGATTATGATGTTGCGATAATAGAT
GAAGCAACTCAGGCAACTATACCGAGCATATTAATACCTCTCAACAAGGTTGATAGGTTTATACTTGCTGGA
GACCACAAGCAACTACCACCAACTATCTTAAGCTTGGAGGCCCAGGAGCTCTCCCACACGCTTTTCGAGGGT
TTAATTGAGAAGTACCCATGGAAGAGCGAAATGCTGACAATTCAGTATAGGATGAATGAGAGGATAATGGAG
TTTCCGAGCAGGGAGTTTTACGATGGAAGAATAGTTGCTGATGAAAGTGTAAAAAACATAACTCTGGCCGAC
CTGGGAATTAAAGTTAATGCTAGTGGAATATGGAGGGACATCCTAGATCCAAACAACGTCCTCGTGTTCATA
GATACTTGCATGCTCGAAAATAGGTTCGAGAGGCAGAGAAGGGGAAGCGAAAGCAGGGAGAATCCCTTGGAG
GCCAAGATAGTGAGCAAAATCGTTGAAAAGCTCTTGGAAAGTGGGGTTAAAGCGGAAATGATGGGAGTGATT
ACACCTTACGATGACCAGAGGGATTTGATAAGCTTGAATGTTCCCGAAGAAGTTGAGGTCAAGACTGTGGAT
GGTTACCAGGGAAGGGAGAAGGAAGTGATAATTCTATCATTTGTCCGCTCTAACAAAGCGGGAGAGATCGGC
TTTCTCAAGGACTTGAGGAGGCTAAACGTGTCCTTAACTAGGGCTAAGAGGAAGCTTATCATGATTGGCGAT
TCCTCAACGCTTTCATCTCACGAAACCTACAGGAGGTTAATCGAGCACGTGAGGGAGAAGGGGTTATATGTT
GTGCTAACGAAGGATAGCATTTGA

FIGURE 29

MIEELFKGLESEIVGLHEIPPKRGEYGEFKFRNEEVNELVKRLGFRLYSHQVKALEKLYS
GKNVVVSTPTASGKSEIFRLFIFDEILSSPSSTFLLIYPTRALINNQMEKFEKENTIFEEICG
KRVRAEVLTGDTEWEKRREIIRSKPNVIFTTPDMLHHHILPRWRDYFWLLKGLRLLVVD
ELHVYRGIFGTNVAYVFKRLFLRLKRLSSSPQILALSATLRNPKEFAEQFFETEFEEVKE
AGSPSPRRIIVMFEPRRFTGEQLIKQIVERLTRKNIKTLVFFDSRKGTERIMRLFLFSDAF
DRITTYKGTLTKRERFLIERDFREGNLTVLLTTNALELGIDIGDLDAVINYGIPSDGLFSLIQ
RFGRAGRDPNRIAINGIILRRNGLDYYYKEHFDELVEGIEKGLVEKIPVNLDNEKIAKKHL
HYAIAELGVVSIKEIEGRWKRFIKTLVEEGYVEVTRNPITGEEEIRLRRPPVYSSIRTASD
ESYFLVVDEPWIRGALQRKRGAELLRFVNYLKVRGMVVEEVDEIEFHRSLLPGMVYLS
RGRPYMAVDKIKIEKFHFVFARPLPIEEEIDTSSSKIENIEILEVKDEKTVGPIKVKFGRLR
VRHEYTGYAVRGRDVERHVKRLEELKDEGILRGEIDIVPYIWESWKFARVLFDTPYIREF
ETEGFWLEFPNDIRIVPEEEFREFFAVASEIDPELAMFLYNRISRKSLFPTLLGATTHYIR
SFILHHAKDKGEEFAFAVKKMIDSKDGIGSGLHAIEPNIIKLAPVVTHVDSREIGGYSYDD
FHGKPVIFIYDGNEGGSGIIRQVYENVEKLMYRSLEHIKKCPCKDGCPACIYSPKCGTFN
EFLDKWMAIRIWEKVLP (SEQ ID NO:)

Figure 30

MLIVVRPGRKKNELEAFIIENPPEKLSQRRNLKADRVVRLIMRDNRLFKALEGSQYLNPKEVERALRNSRIV
LVNANEWEEYFKKRLMNKRVEKADICRLCLLNGKITVLTEGNRIRYRDEYICESCAEEELKRELRFRFNSIG
MLEQAKKLLERFRDLDKVISIFDPSFDPTKHPEITKWDELKAKHIRVEKMHIDELNIPEEFKKVLKAEGINE
LLPVQVLAIKNGLLEGENLLVVSATASGKTLIGELAGIPKALKGKKMLFLVPLVALANQKYEDFKRRYSKLG
LKVAIRVGMSRIKTKEEPIVLDTGTDAHIIVGTYEGIDYLLRAGKKIGNVGTVVIDEIHMLDDEERGARLDG
LIARLRKLYSNAQFIGLSATVGNPQELARKLGMKLVLYDERPVDLERHLIIARNESEKWRYIAKLCKAEAMR
KSEKGFKGQTIVFTFSRRRCHELASFLTGQGLKAKAYHSGLPYVQRKLTEMEFQAQMIDVVVTTAALGAGVD
FPASQVIFESLAMGNKWITVREFHQMLGRAGRPQYHEKGKVYIIVEPGKKYSAQMEGTEDEVALKLLTSPIE
PVIVEWSDEFEEDNVLAHACVFNRLKVIEEVQSLCLGANQSAKNVLEKLMEKGLVKIYGDKVEATPYGRAVS
MSFLLPREAEFIRDNLESTDPIEIAIKLLPFENVYLPGSLQREIESAVRGKISSNIFSSSFASVLEELDKII
PEISPNAAERLFLIYQDFFNCPEQDCTEFAMERIGRKIIDLRREGYEPSKISEHFRKVYALILYPGDVFTWL
DGIVRKLEAIERIARVFNKRRVVEDTIRVRREIEEGKILKGERR

FIGURE 31

MHKYFFPLPATKSTFLLPADLTTANPCFSKSLINSLSAWAPFLYIQCFSYLPLINFLNSLT
YPLEMHILIKKAIKERFGKLNALQQLAFHKIRGEGKSVLIIAPTGSGKTEAAVIPILDAILRE
NLKPIAAIYIAPLKALNRDLLERLKWWEEKTGVIIEVRHGDTPTSKRLKQVKNPPHLLITTP
EMLPAILTTKSFRPYLKNTKFIVIDEIGELIENKRGTQLILNLKRLELITEDKPIRIGLSATIGS
EEKVRLWMEADEVVKPRLKKKYKFTVLYPQPIPEDEKLAEELKVPIEVATRLRVVWDIVE
KHKKVLIFVNTRQFAEILGHRLKAWGKPVEVHHGSLSREARIEAEKKLKEGKIKALICTSS
MELGIDIGDVDAVIQYMSPRQVNRLVQRAGRSKHRLWETSEAYIITTNVEDYLQSLAIAK
LALEGKLEDVNPYENALDVLAHFIVGLTIEYRNVNITEPYSLAKSTYPYRKLSWEDYQKV
LEILEEARIIRRDGDAIKLGKNAFKYYFENLSTIPDEISYAVIDIASGKSVGRLDENFVTELE
ESMEFIMHGRSWIVLEINEKERIIKVKESNNLESALPSWEGELIPVPLEVAEFVGKLKREL
LWDKERALKLLEGVEFNKEELEVAISQLVESEPVASDRDIIIESYPKFVIIHADFGNKINEG
LTRFISVFLSARYGNIFLPRSQAHGIIIRSPFRLNPEEIKEILLMKAEVGDIVARGIRDTPIYR
WKMSAIAKRFGALRRDARIKKVERLFEGTIIEKETFNEIYHDKIDIDKTEKILEKIRKGEIRM
KTLFREEITPLSSSLATLGGEFLIRDILTQEEVEEIFREKLLDAELVMVCTNCGFSWRTKV
RRVMDRVNELSCPKCDSKMIAPLHPKDSETFISALKKLKRGEKLSREEEKYYLRGLKAA
DLLKAYGKDALLALATYGVGVESATRILRDYRGKSLIKALIEAEKHYIQTRKFWE
(SEQ ID NO:)

FIGURE 32

VMLLRRDLIQPRIYQEVIYAKCKETNCLIVLPTGLGKTLIAMMIAEYRLTKYGGKVLMLAP
TKPLVLQHAESFRRLFNLPPEKIVALTGEKSPEERSKAWARAKVIVATPQTIENDLLAGRI
SLEDVSLIVFDEAHRAVGNYAYVFIAREYKRQAKNPLVIGLTASPGSTPEKIMEVINNLGI
EHIEYRSENSPDVRPYVKGIRFEWVRVDLPEIYKEVRKLLREMLRDALKPLAETGLLESS
SPDIPKKEVLRAGQIINEEMAKGNHDLRGLLLYHAMALKLHHAIELLETQGLSALRAYIKK
LYEEAKAGSTKASKEIFSDKRMKKAISLLVQAKEIGLDHPKMDKLKEIIREQLQRKQNSKI
IVFTNYRETAKKIVNELVKDGIKAKRFVGQASKENDRGLSQREQKLILDEFARGEFNVLV
ATSVGEEGLDVPEVDLVVFYEPVPSAIRSIQRRGRTGRHMPGRVIILMAKGTRDEAYYW
SSRQKEKIMQETIAKVSQAIKKQKQTSLVDFVREKESEKTSLDKWLKKEKEEATEKEEK
KVKAQEGVKVVVDSRELRSEVVKRLKLLGVKLEVKTLDVGDYIISEDVAIERKSANDFIQ
SIIDGRLFDQVKRLKEAYSRPIMIVEGSLYGIRNVHPNAIRGAIAAVTVDFGVPIIFSSTPE
ETAQYIFLIAKREQEEREKPVRIRSEKKALTLAERQRLIVEGLPHVSATLARRLLKHFGSV
ERVFTASVAELMKVEGIGEKIAKEIRRVITAPYIEDEE (SEQ ID NO:)

FIGURE 33

LKGLFRDVILHNPHLFVYSYSDKGIIPFKHQFQTLYHAMLMRPVRLMIADEIGLGKTIQAL
LIAKYLDFRGEIEKALIVVPKVLREQWREEVKRILEEAPEVIENGSEIEWKLKRPRKYFIISI
DLAKRYTEEILRQKWDLVIVDEVHNATLGTQRYEFLKELTKNKDLNVIFLSATPHRGNNR
DYLARLRLLDPTIPEEISPMHERKIYMKSRGTLVLRRTKKVVNELEGEVFKKCHFGAVVV
EVSREEREFFEELNRALFELIKDQADYSPLTLLAVIIRKRASSSYEAALKTLTRIVESAYIS
GQERARGVESYIEKIFRMGYEELEIEEFNEIDDAIHKIIDEYRGFLTEEQLERLRRVLELG
KKIGSKDSKLEVISDIVAYHIRNGEKVIIFTEFRDTLEYVLERLPDILRRKHGIVLEKDDIAK
LHGGMKSEEIEREINKFHERANLLVSTDVASEGLNLHVASVVINYEAPWSPIKLEQRVG
RIWRLNQTRETKAYTIFLATETDLDVLNNLYRKIMNIKEAVGSGPIIGRPIFEGDFENLWN
EGAEEENREVSEYELILASIKGELKGYAGALVRTLRILKQKVEGAVPVNPAGSIRRELEIIL
EDTPDVEVLKKIVNRNVPNPFRLVRGLLREAEGIEGIRVLVKGYDGSMDVYYAIFYDED
GREIYRYPILAENGKYLVGFNLLKRISEVLSKEYKVVRGASEEVDYKVKTLVMDNIYNLIV
KKYLEYDSLNIKEGKIFKRLKVEIKKALEVKGISEEEFEVIKRVPPEIMEVLGLDSTKIELPT
NEYLKIFERNFVPLDKILESEKKAMEIVMELEKSRGYNVEDVSLREHYDIRAFTDGEEKYI
EVKGHYPMLLLAELTEKEFEFAQKNEDKYWIYIVSNIAKDPVIVKIYKPFSQDRRVFVVK
NGEDVEVNINIEIKKKDRHLLKLS (SEQ ID NO:)

FIGURE 34

VITLELHPSEIARYFELEECSHYFSNLLLRKRGELQEFEPIIRRKEIETIELAKWGDEFELS
LLQEFKKGEALKKLGVKELPRFYGFLTENDTPVRKFFEKYFKDGIIVEEDPDKLLEIINSE
KSAVIYQAPLKGRIGKFDVSGRADFIIKVGKTLYLLEAKFTKEEKFYHRIQAIIYAHLLSQMI
EGYEIKLAVVTKENFPIPSNFLRFPGDVEELKITLEEKLGGILREQELWIDARCTTCPFEA
LCLSKALEERSLGLLSLPPGIIRILKEEGIKDLKDMAKLFEFKENSPTNFEEPSIKDPKKTQ
EIAKRTGINLLKLSRIAQAILKYLDEGETTPLFIPRTGYNLPMDERVGDVEPSYYPPRSLV
KVFFYVQTSPITDTIIGISALVKNRQNGERIIVKFVDEPPIEVSDAQEKERMLLIEFFRDVID
AVKSLSPTDKVYLHMYFYNRKQRDDLMDAVKRHKEIRENNAVMALLSLRRAIDWESFSI
IKDEIIRRHALPLSPGLGFVTVATQFGYRWRRNKTFARMLEVVARRENGKINLKTLLNIS
ETGIGPEYYPIIDRDNEGIPFTLFWSALVKLATEEDNSRIKRDIRDILSQMVEALKTIEERIP
EQYKDAFVKKEGIPKEDLENFDIKKEELADILLEYLQLEFDARFRERSEYYRLPLSIRAYS
EESALIKIENIEKKKNDCLLFGKIVLIDENGRIKEYNPKEVLIDIDEGSLVVVTPKKFLDKLR
RDPVQRISKSPLGIVEAIDHETGKVVIRLIRVSPGRFTLKHSKFSCKNGLLTITYPEGEVK
VTPGEIVIVDPSVDDIGMERAYNVLSEISQGELKHEIYQKVKAIYEGNTESRYEVNIWKKK
HIEEFLSRVKKINEEQKKFAIDINNFLVTLQGPPGTGKTSGAIAPAILARAYSMVKDKKNG
LFVVTGVSHRAVNEALIKTLKLKKELENTLKELRKIDLIRAVSGEEAIKIIKEELEREIKDDV
DRIRFTAQEITHSSKQRSLDKYFANSGTVRIVFGTPQTLNKLMKNTKEVELVVIDEASMM
DLPMFFLSTKVCKGQVLLVGDHRQMEPIQVHEWQLEDRKTFEEHYPFLSALNFIRFLR
GELDERELKKFKRILGREPPEWKKDKNEVLPLYRLVRTYRLPQEIADLLSDAIYRADGIK
LISEKKKRRKIIARHKDEFLSIVLDDRYPFVLILHDEGNSTKINELEAKIVEKIIKRVENIDIG
VVVPYRAQKRLIASLIDSAQVDTVERFQGGEKSLIVISMTSSDPRIPGKGF
(SEQ ID NO:)

Figure 35

MNIKSFINRLKELVEIEREAEIEAMRLEMKRLSGVERERLGRAILSLNGKIVGEELGYFLVKYGRNKEIKTE
ISVGDLVVISKRDPLKSDLLGTVVEKGKRFIVVALEPVPEWALRDVRIDLYANDITFKRWIENLDRVRKAGK
KALEFYLGLDEPSQGEEVSFEPFDKSLNPSQRKAIAKALGSEDFFLIHGPFGTGKTRTLVELIRQEVKRGNK
VLATAESNVAVDNLVERLAKDGVKIVRVGHPSRVSRHLHETTLAYLITQHELYGELRELRVIGQSLAEKRDT
YTKPTPKFRRGLSDAEIIKLAEKGRGARGLSARLIKEMAEWIKLNRQVQKAFEDARKLEERIARDIIREADV
VLTTNSSAALDVVDATDYDVAIIDEATQATIPSILIPLNKVDRFILAGDHKQLPPTILSLEAQELSHTLFEG
LIEKYPWKSEMSTIQYRMNERIMEFPSREFYDGRIVADESVKNITLADLGIKVNASGIWRDILDPNNVLVFI
DTCMLENRFERQRRGSESRENPLEAKIVSKIVEKLLESGVKAEMMGVITPYDDQRDLISLNVPEEVEVKTVD
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VLTKDSI

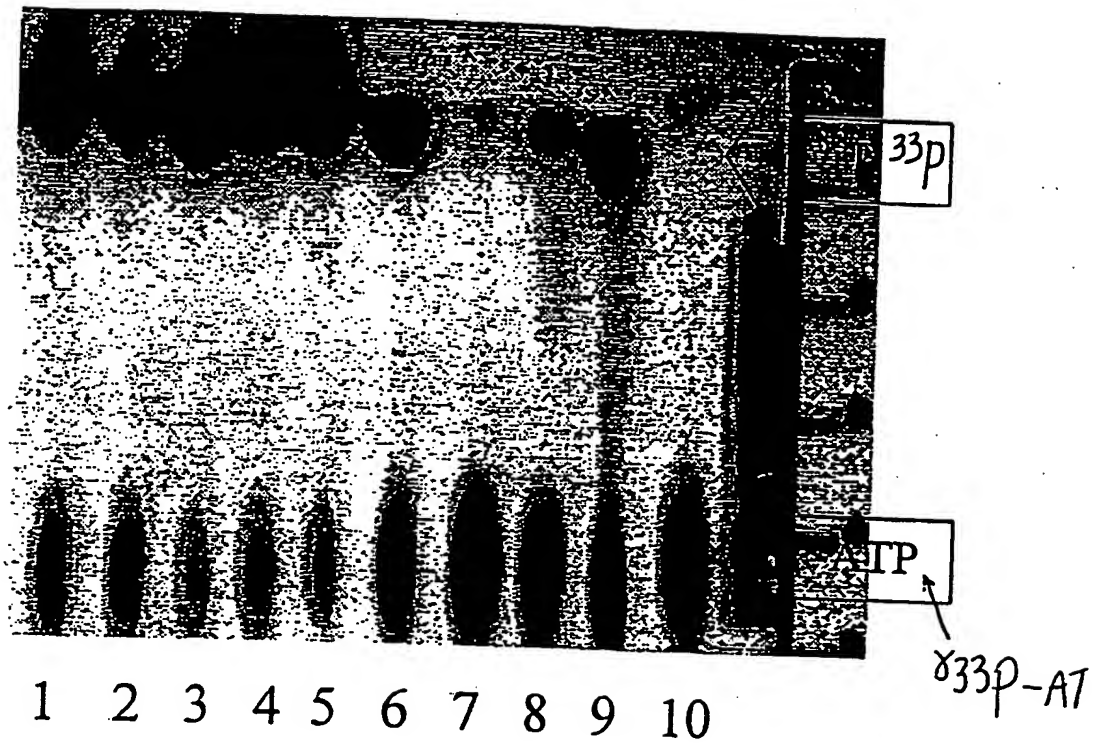

Figure 36.
ATPase Assay from Phage Induced Helicases

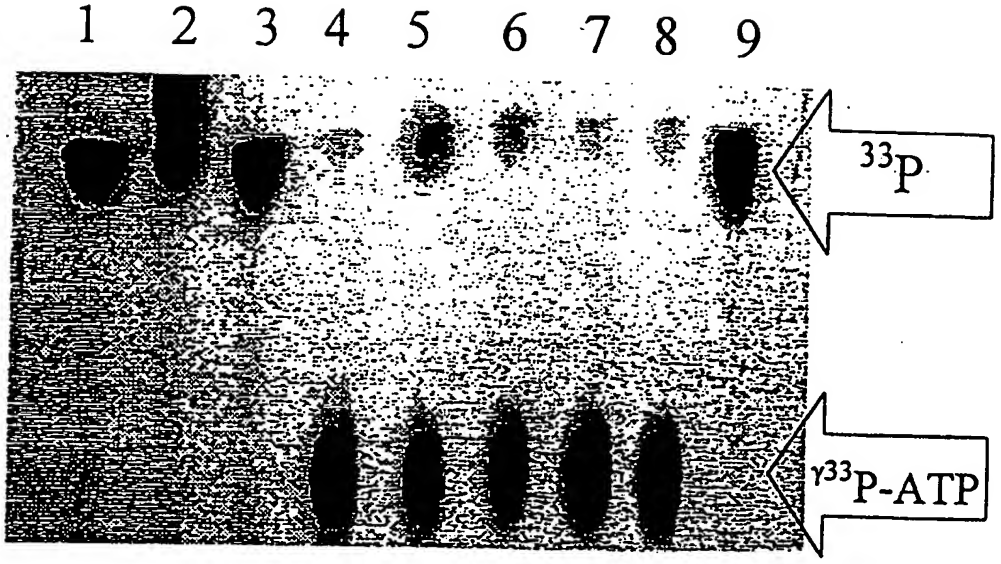

Figure 37.
ATPase Assay from IPTG Induced Helicases

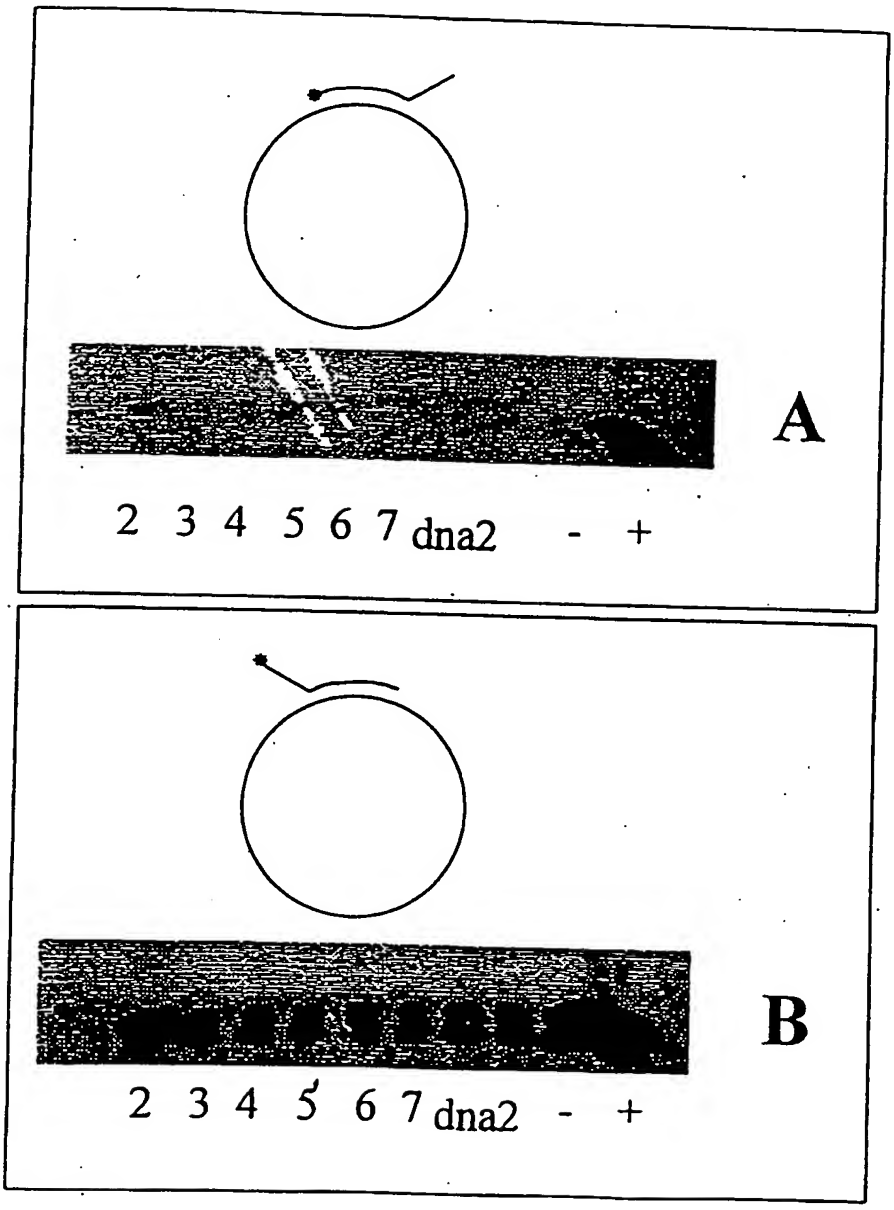

Figure 38.
Helicase Displacement of Bound Oligonucleotides

Figure 39

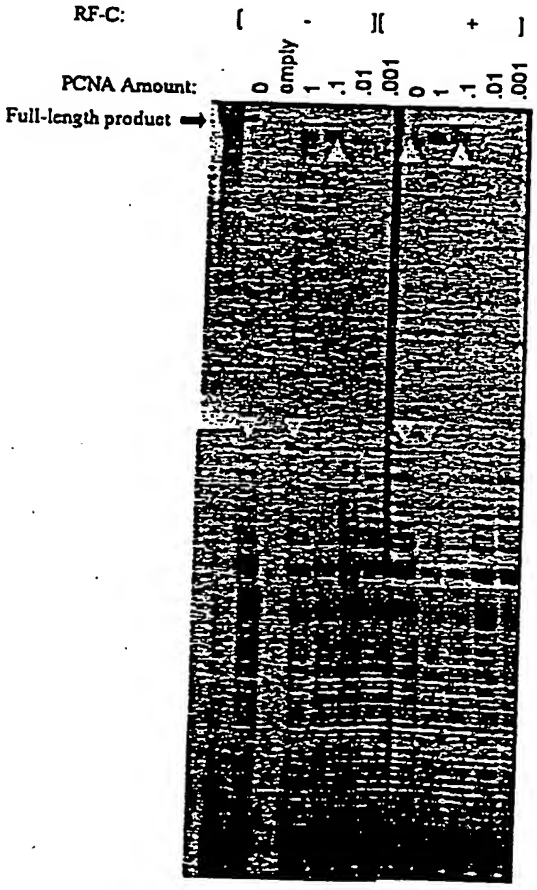
RF-C:
[-][+]
PCNA Amount:
0
empty
1
.1
.01
.001
0
1
.1
.01
.001
Full-length product

Figure 40

ATGAGGGTTGATGAGCTGAGAGTTGATGAGAGGATAAAGAGTACTTTGAAGGAGAGAGGTATCGAATCCTTT
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GCCAGCGGAAAAACACTAATTGCTGAGATTGCCATGGTTCATAGGATTTTGACCCAGGGAGGAAAGGCTGTA
TACATAGTCCCGCTGAAGGCCTTGGCTGAAGAAAAGTTTCAGGAGTTCCAGGATTGGGAGAAGATTGGGTTA
AGAGTAGCGATGGCCACTGGGGATTACGACTCAAAGGATGAGTGGTTGGGGAAATACGACATAATCATTGCG
ACGGCTGAGAAGTTTGATTCCCTTTTAAGGCATGGCTCAAGTTGGATTAAGGATGTGAAGATTTTAGTTGCT
GACGAGATTCATTTGATTGGTTCAAGAGACAGAGGAGCTACGCTTGAAGTTATCCTAGCTCATATGCTCGGA
AAGGCCCAAATAATTGGACTCTCTGCAACGATAGGAAATCCAGAGGAGCTTGCGGAGTGGTTAAATGCCGAG
CTAATAGTCAGTGACTGGAGGCCCGTTAAGCTTAGAAGGGGAGTTTTTTACCAAGGCTTTGTTACCTGGGAA
GATGGAAGTATAGACAGGTTTTCCTCCTGGGAAGAGTTAGTTTACGATGCAATTAGGAAGAAGAAAGGAGCG
CTAATTTTTGTAAACATGAGAAGGAAGGCTGAGAGAGTAGCTTTGGAGCTTTCTAAAAAAGTTAAGTCTCTC
CTCACGAAACCTGAGATTAGAGCTTTAAATGAATTGGCTGATTCCCTCGAGGAAAATCCCACAAATGAAAAG
CTAGCTAAGGCCATTAGGGGTGGAGTTGCGTTCCACCACGCTGGTCTTGGGAGAGATGAGAGGGTTCTCGTG
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CCAGCGTTTAGGGTGATTATAAGGGATATTTGGAGGTACTCTGACTTTGGAATGGAGAGAATTCCGATAATC
GAGGTTCACCAAATGCTTGGGAGAGCTGGAAGGCCGAAGTATGATGAGGTTGGGGAGGGAATAATAGTTTCT
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TCCAACGAGAGTAATTTGAGAAGTCAAGTTTTGGCCCTAATAGCGACCTTTGGCTATTCAACTGTGGAGGAG
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AGGAACATACTCTACTTCCTCCTAGAGAATGAGTTCATAGAGATATCCTTAGAGGATAAAATAAGGCCGCTT
TCCCTGGGAATTAGGACTGCAAAGCTTTATATCGATCCCTATACGGCCAAGATGTTCAAGGATAAAATGGAG
GAAGTTGTTAAAGATCCAAATCCTATAGGAATATTTCACTTAATCTCCCTAACTCCGGATATAACCCCCTTC
AACTACTCAAAGAGAGAATTTGAAAGGCTCGAAGAGGAATACTACGAATTCAAGGATAGGTTATACTTTGAC
GATCCCTACATTTCGGGTTACGACCCCTACCTAGAGAGGAAGTTCTTCAGAGCTTTCAAAACTGCACTAGTG
CTTCTGGCATGGATAAATGAAGTCCCTGAGGGAGAAATAGTTGAAAAGTACTCGGTGGAACCTGGGGACATC
TATAGGATAGTTGAGACGGCTGAGTGGCTGGTGTACTCTCTAAAGGAAATTGCAAAAGTTCTTGGAGCTTAT
GAGATCGTTGATTATCTTGAAACATTGAGGGTTAGGGTCAAGTATGGGATTAGGGAGGAATTGATTCCCCTA
ATGCAACTCCCGTTGGTTGGAAGAAGGAGAGCTAGAGCTCTTTACAATAGCGGATTTAGAAGTATAGAGGAT
ATATCTCAAGCGAGGCCAGAAGAGCTTTTGAAAATCGAGGGGATAGGGGTCAAGACCGTTGAGGCTATCTTC
AAGTTTCTTGGTAAGAATGTGAAAATTTCGGAGAAACCTAGAAAAAGTACCCTTGATTACTTTCTCAAATCT
TGA

Figure 41

MRVDELRVDERIKSTLKERGIESFYPPQAEALKSGILEGKNALISIPTASGKTLIAEIAMVHRILTQGGK
AVYIVPLKALAEEKFQEFQDWEKIGLRVAMATGDYDSKDEWLGKYDIIIATAEKFDSLLRHGSSWIKDVK
ILVADEIHLIGSRDRGATLEVILAHMLGKAQIIGLSATIGNPEELAEWLNAELIVSDWRPVKLRRGVFYQ
GFVTWEDGSIDRFSSWEELVYDAIRKKKGALIFVNMRRKAERVALELSKKVKSLLTKPEIRALNELADSL
EENPTNEKLAKAIRGGVAFHHAGLGRDERVLVEENFRKGIIKAVVATPTLSAGINTPAFRVIIRDIWRYS
DFGMERIPIIEVHQMLGRAGRPKYDEVGEGIIVSTSDDPREVMNHYIFGKPEKLFSQLSNESNLRSQVLA
LIATFGYSTVEEILKFISNTFYAYQRKDTYSLEEKIRNILYFLLENEFIEISLEDKIRPLSLGIRTAKLY
IDPYTAAMFKDAMEEVVKDPNPIGIFHLISLTPDITPFNYSKREFERLEEEYYEFKDRLYFDDPYISGYD
PYLERKFFRAFKTALVLLAWINEVPEGEIVEKYSVEPGDIYRIVETAEWLVYSLKEIAKVLGAYEIVDYL
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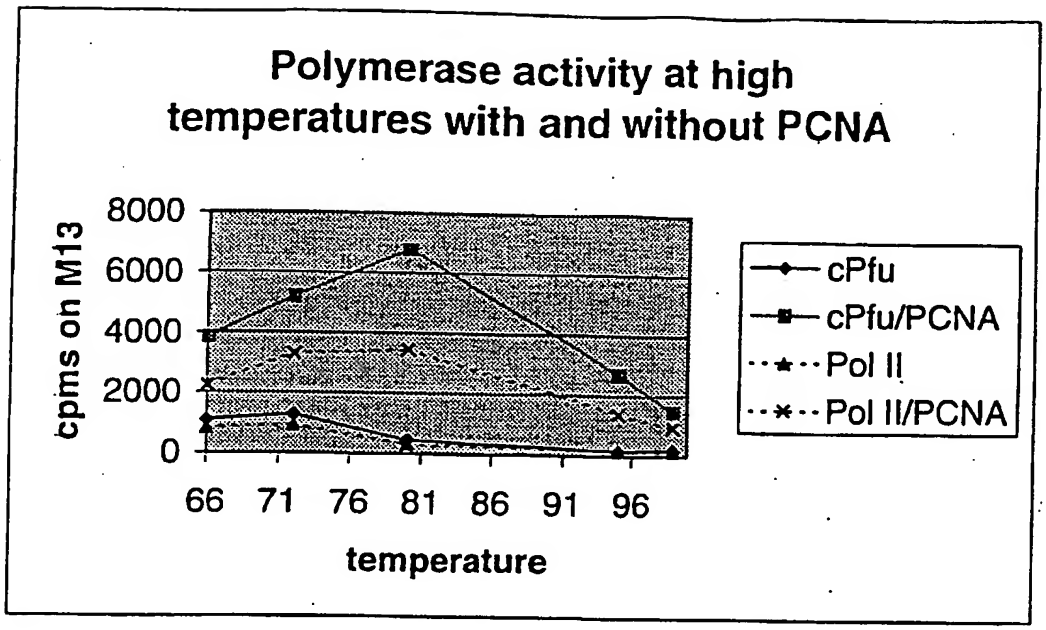
Polymerase activity at high temperatures with and without PCNA
cpms on M13
8000
6000
4000
2000
0
66
71
76
81
86
91
96
temperature
cPfu
cPfu/PCNA
Pol II
Pol II/PCNA

Figure 43

ATGAGGGTTGATGAGCTGAGAGTTGATGAGAGGATAAAGAGTACTTTGAAG
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GGGATATTGGAAGGTAAGAATGCATTAATTTCAATTCCAACGGCCAGCGGA
AAAACACTAATTGCTGAGATTGCCATGGTTCATAGGATTTTGACCCAGGGAG
GAAAGGCTGTATACATAGTCCCGCTGAAGGCCTTGGCTGAAGAAAAGTTTC
AGGAGTTCCAGGATTGGGAGAAGATTGGGTTAAGAGTAGCGATGGCCACTG
GGGATTACGACTCAAAGGATGAGTGGTTGGGGAAATACGACATAATCATTG
CGACGGCTGAGAAGTTTGATTCCCTTTTAAGGCATGGCTCAAGTTGGATTAA
GGATGTGAAGATTTTAGTTGCTGACGAGATTCATTTGATTGGTTCAAGAGAC
AGAGGAGCTACGCTTGAAGTTATCCTAGCTCATATGCTCGGAAAGGCCCAA
ATAATTGGACTCTCTGCAACGATAGGAAATCCAGAGGAGCTTGCGGAGTGG
TTAAATGCCGAGCTAATAGTCAGTGACTGGAGGCCCGTTAAGCTTAGAAGG
GGAGTTTTTTACCAAGGCTTTGTTACCTGGGAAGATGGAAGTATAGACAGGT
TTTCCTCCTGGGAAGAGTTAGTTTACGATGCAATTAGGAAGAAGAAAGGAG
CGCTAATTTTTGTAAACATGAGAAGGAAGGCTGAGAGAGTAGCTTTGGAGC
TTTCTAAAAAAGTTAAGTCTCTCCTCACGAAACCTGAGATTAGAGCTTTAAAT
GAATTGGCTGATTCCCTCGAGGAAAATCCCACAAATGAAAAGCTAGCTAAG
GCCATTAGGGGTGGAGTTGCGTTCCACCACGCTGGTCTTGGGAGAGATGA
GAGGGTTCTCGTGGAGGAGAACTTTAGAAAGGGTATAATAAAGGCCGTAGT
TGCCACCCCAACACTTTCGGCGGGAATTAACACTCCAGCGTTTAGGGTGAT
TATAAGGGATATTTGGAGGTACTCTGACTTTGGAATGGAGAGAATTCCGATA
ATCGAGGTTCACCAAATGCTTGGGAGAGCTGGAAGGCCGAAGTATGATGAG
GTTGGGGAGGGAATAATAGTTTCTACAAGCGATGATCCGAGAGAGGTAATG
AATCACTACATATTTGGAAAGCCTGAAAAACTGTTCTCCCAGCTCTCCAACG
AGAGTAATTTGAGAAGTCAAGTTTTGGCCCTAATAGCGACCTTTGGCTATTC
AACTGTGGAGGAGATTTTGAAGTTCATCTCAAACACATTCTATGCTTATCAAA
GGAAGGACACATACTCTTTAGAGGAGAAGATAAGGAACATACTCTACTTCCT
CCTAGAGAATGAGTTCATAGAGATATCCTTAGAGGATAAAATAAGGCCGCTT
TCCCTGGGAATTAGGACTGCAAAGCTTTATATCGATCCCTATACGGCCAAGA
TGTTCAAGGATAAAATGGAGGAAGTTGTTAAAGATCCAAATCCTATAGGAAT
ATTTCACTTAATCTCCCTAACTCCGGATATAACCCCCTTCAACTACTCAAAGA
GAGAATTTGAAAGGCTCGAAGAGGAATACTACGAATTCAAGGATAGGTTATA
CTTTGACGATCCCTACATTTCGGGTTACGACCCCTACCTAGAGAGGAAGTTC
TTCAGAGCTTTCAAAACTGCACTAGTGCTTCTGGCATGGATAAATGAAGTCC
CTGAGGGAGAAATAGTTGAAAAGTACTCGGTGGAACCTGGGGACATCTATA
GGATAGTTGAGACGGCTGAGTGGCTGGTGTACTCTCTAAAGGAAATTGCAA
AAGTTCTTGGAGCTTATGAGATCGTTGATTATCTTGAAACATTGAGGGTTAG
GGTCAAGTATGGGATTAGGGAGGAATTGATTCCCCTAATGCAACTCCCGTT
GGTTGGAAGAAGGAGAGCTAGAGCTCTTTACAATAGCGGATTTAGAAGTAT
AGAGGATATATCTCAAGCGAGGCCAGAAGAGCTTTTGAAAATCGAGGGGAT
AGGGGTCAAGACCGTTGAGGCTATCTTCAAGTTTCTTGGTAAGAATGTGAAA
ATTTCGGAGAAACCTAGAAAAAGTACCCTTGATTACTTTCTCAAATCTTGA

Figure 44

MRVDELRVDERIKSTLKERGIESFYPPQAEALKSGILEGKNALISIPTASGKTLIAE
IAMVHRILTQGGKAVYIVPLKALAEEKFQEFQDWEKIGLRVAMATGDYDSKDEW
LGKYDIIIATAEKFDSLLRHGSSWIKDVKILVADEIHLIGSRDRGATLEVILAHMLG
KAQIIGLSATIGNPEELAEWLNAELIVSDWRPVKLRRGVFYQGFVTWEDGSIDR
FSSWEELVYDAIRKKKGALIFVNMRRKAERVALELSKKVKSLLTKPEIRALNELA
DSLEENPTNEKLAKAIRGGVAFHHAGLGRDERVLVEENFRKGIIKAVVATPTLSA
GINTPAFRVIIRDIWRYSDFGMERIPIIEVHQMLGRAGRPKYDEVGEGIIVSTSDD
PREVMNHYIFGKPEKLFSQLSNESNLRSQVLALIATFGYSTVEEILKFISNTFYAY
QRKDTYSLEEKIRNILYFLLENEFIEISLEDKIRPLSLGIRTAKLYIDPYTAKMFKDK
MEEVVKDPNPIGIFHLISLTPDITPFNYSKREFERLEEEYYEFKDRLYFDDPYISG
YDPYLERKFFRAFKTALVLLAWINEVPEGEIVEKYSVEPGDIYRIVETAEWLVYSL
KEIAKVLGAYEIVDYLETLRVRVKYGIREELIPLMQLPLVGRRRARALYNSGFRSI
EDISQARPEELLKIEGIGVKTVEAIFKFLG KNVKISEKPRKSTLDYFLKS

Figure 45. Nucleic acid sequence of *P. furiosus mcm* gene (intein present)

GTGGACAGGGAGGAGATGATTGAGAGATTTGCAAACTTCCTTAGGGAGTATACAG
ACGAAGATGGTAACCCAGTATACAGAGGTAAAATAACTGATTTACTTACAATAACAC
CCAAGAGGTCTGTTGCAATAGACTGGATGCACCTAAATTCCTTTGACTCAGAGCTA
GCTCATGAAGTTATAGAGAACCCCGAAGAAGGAATAAGTGCCGCAGAAGATGCAA
TCCAGATTGTATTACGAGAAGACTTCCAAAGAGAAGACGTGGGAAAAATACACGCA
AGGTTTTATAATTTGCCAGAAACCCTAATGGTCAAAGACATTGGGGCAGAGCACAT
CAACAAGTTAATTCAAGTAGAAGGAATCGTGACGAGAGTAGGAGAAATTAAGCCCT
TTGTCTCTGTGGCAGTTTTCGTATGTAAGGACTGCGGTCATGAAATGATAGTGCCT
CAAAAACCCTATGAAAGCCTTGAAAAAGTTAAGAAGTGCGAACAATGTGGAAGCAA
AAATATAGAACTAGATGTTAACAAGAGCTCCTTCGTAAACTTCCAGAGCTTTAGGAT
TCAGGATAGACCAGAAACCCTAAAAGGAGGAGAAATGCCAAGGTTTATCGATGGTA
TTCTGCTTGATGACATAGTGGATGTAGCCCTCCCAGGAGACAGAGTTATTGTAACA
GGAATTTTGAGAGTCGTTCTTGAAAAGAGAGAGAAAACTCCAATATTTAGAAAAATC
CTCGAGGTAAATCACATTGAACCTGTTAGTAAAGAGATACAAGAATTAGAAATTTCT
CCAGAAGAGGAGCAGATAATAAAGGAGCTAGCAAAAAGAAAAGACATAGTAGATG
CAATAGTTGATTCAATAGCTCCTGCCATATATGGATACAAAGAAGTCAAGAAGGGA
ATAGCACTTGCCCTGTTTGGAGGAGTTTCAAGAAAGTTACCTGATGGAACTAGGCT
TAGAGGAGATATACATGTCCTCCTGGTCGGAGACCCAGGAGTTGCAAAGAGCCAG
ATTTTAAGGTATGTGGCAAACCTCGCTCCTAGGGCCATTTACAC
TTCAGGAAAAAGTAGTTCCGCAGCAGGTCTT

TGTGTAGCTCCCGATTCTTTAGTGGTAGTGAATGACAAAGTTCAAGAAATAGGAAA
GCTAACGGAAGAATGGGGAAGAGAAGTAGGCTTCCTAGAATACTCAAGTGGGATT
TTCTATGCTCCTTACCTGGGAAGAGGAATATCCCTAGATTTAGTAACAGGGAAAGT
CAAACCTTCAGTTGTTAGCAAGGTTTGGAAGTTAAAATCCCCAGAAGAATTAGTTAC
AATAAAGACCATTACTGGAAAAGAGATAACAGTAACTCCTGAGACAAAACTTCTGA
CCTTCAATGGGACACTTGAATGGAAAGAAGCTGGAAAAATAAAACCTGGAGATTAC
GTCCTAACGGTTAAAAAGTTACATATCAATGGGAAACAAGAAACTTTAGATGAAAAG
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GAGAGGACAATTTCCGCTTATCTAAAGGGAATATTTGACAAAGTCGGAAGACTCGT
CGGAGATACAGCGGTCATTAAAGTCGATAAAGATATGGCAAAGAGGCTACAGATTT
TATTGCTAAGGCTTGGAATAGTTTCCTCGGTAGATGAGACAGGAAAAGTCATCATT
GGAAGGGAGTACATCCAGAAAATCTTAGGGTACAACGTTAGCGTCGTGACCCATG
AAGTGGAGCTATTTAGAGAGTTTATAGCTGAAATATCTAAGTTCTATGGAACCAGTG
AAGAGGATGTCTACAGTTCCCTCCATGAAAAAGGAGAACTCGATATAGGGACAGTT
CCAGTAGAGCTCCCAGAGGGCTTAAGAGAAGAAATAAATCGTGAAAGAGCAACTTA
CAGTGAACTTGTGAAAATTGCCCAGGAAATAAAAGATGAAAAACTCTACAATAAACT
TGCGTGGATTTTAAGTGAAGTTACGGAAGAAGAGGCAAAAATTAAGGAAAAAGTTA
ACACTCTAAAGGTCATACTCTCCTCAGATTTGATACCAGAAAGAGTAGAATCTGTAA
AGATTATCAAAAGTCCATACCCCTACGTTTATGACCTTACAGTTGAAGGTTCTCACA
GCTTCATAGCAAATGGCTTTGTAGTCCACAAT

Figure 45 cont.

ACTGCTGCAGCAGTTAGGGATGAGTTCACGGGAGGATGGGTTTTGGAGGCGGGA
GCTTTAGTCCTTGCAGATGGGGGTTATGCTCTAATCGACGAGCTCGACAAGATGA
GCGACAGGGATAGGAGCGTGATACATGAAGCCTTAGAACAACAGACAATAAGCAT
TTCAAAAGCAGGGATTACGGCAACTCTAAACGCTAGAACTACAGTCATTGCGGCTG
CAAATCCGAAACAGGGAAGATTTAATAGAATGAAAAATCCATTCGAGCAAATTGAC
CTTCCCCCTACACTTCTAAGTAGATTTGACCTAATATTTGTGTTAATTGATGAGCCC
GATGACAAAATTGACAGTGAAGTTGCCAGACACATCTTAAGGGTCAGAAGGGGAG
AAAGTGAAGTCGTGGCCCCAAAAATACCTCATGAAATTCTAAGGAAGTACATCGCT
TATGCAAGGAAGAATATTCATCCCGTTATAAGTGAAGAAGCTATGGAAGAGATAGA
GAAGTACTATGTGAGAATGAGAAAGAGTGTAAAGAAGACAAAGGGAGAAGAAGAG
GGGATACCACCAATCCCAATAACAGCTAGACAGCTCGAGGCCCTCATTAGATTAAG
CGAAGCTCATGCAAGGATGAGGCTAAGCCCAATAGTAACAAGGGAAGATGCAAGA
GAAGCAATAAAACTGATGGAATACACGCTAAAGCAAATTGCAATGGATGAGACCGG
GCAAATTGACGTGACAATTCTAGAATTAGGTCAGAGCGCAAGAAAGCTCAGTAAAA
TAGAGAAAATACTGGATATCATTGAAAAGCTTCAGAAGACCAGCGAAAGAGGCGCC
CACGTTAATGATATCTTAGAAGAAGCAAAGAAAGCAGGAATAGAGAAGCAGGAAGC
AAGAGAAATCCTTGAAAAACTTTTGGAGAAGGGTCAAATATATATGCCAGAGAGTG
GTTACTACAAAACCGTCTGA

Figure 46. Translated amino acid sequence of *P. furiosus* MCM protein (intein present)

VDREEMIERFANFLREYTDEDGNPVYRGKITDLLTITPKRSVAIDWMHLNSFDSELAHE
VIENPEEGISAAEDAIQIVLREDFQREDVGKIHARFYNLPETLMVKDIGAEHINKLIQVEGI
VTRVGEIKPFVSVAVFVCKDCGHEMIVPQKPYESLEKVKKCEQCGSKNIELDVNKSSF
VNFQSFRIQDRPETLKGGEMPRFIDGILLDDIVDVALPGDRVIVTGILRVVLEKREKTPIF
RKILEVNHIEPVSKEIQELEISPEEEQIIKELAKRKDIVDAIVDSIAPAIYGYKEVKKGIALAL
FGGVSRKLPDGTRLRGDIHVLLVGDPGVAKSQILRYVANLAPRAIYTSGKSSSAAGL

CVAPDSLVVVNDKVQEIGKLTEEWGREVGFLEYSSGIFYAPYLGRGISLDLVTGKVKPS
VVSKVWKLKSPEELVTIKTITGKEITVTPETKLLTFNGTLEWKEAGKIKPGDYVLTVKKLH
INGKQETLDEKLAYKRGLSLSDPLEFFSSSERTISAYLKGIFDKVGRLVGDTAVIKVDKD
MAKRLQILLLRLGIVSSVDETGKVIIGREYIQKILGYNVSVVTHEVELFREFIAEISKFYGT
SEEDVYSSLHEKGELDIGTVPVELPEGLREEINRERATYSELVKIAQEIKDEKLYNKLAW
ILSEVTEEEAKIKEKVNTLKVILSSDLIPERVESVKIIKSPYPYVYDLTVEGSHSFIANGFV
VHN

TAAAVRDEFTGGWVLEAGALVLADGGYALIDELDKMSDRDRSVIHEALEQQTISISKAG
ITATLNARTTVIAAANPKQGRFNRMKNPFEQIDLPPTLLSRFDLIFVLIDEPDDKIDSEVA
RHILRVRRGESEVVAPKIPHEILRKYIAYARKNIHPVISEEAMEEIEKYYVRMRKSVKKTK
GEEEGIPPIPITARQLEALIRLSEAHARMRLSPIVTREDAREAIKLMEYTLKQIAMDETGQ
IDVTILELGQSARKLSKIEKILDIIEKLQKTSERGAHVNDILEEAKKAGIEKQEAREILEKLL
EKGQIYMPESGYYKTV

Figure 47. Translated amino acid sequence of *P. furiosus* MCM (intein removed)

VDREEMIERFANFLREYTDEDGNPVYRGKITDLLTITPKRSVAIDWMHLNSFDSELAHE
VIENPEEGISAAEDAIQIVLREDFQREDVGKIHARFYNLPETLMVKDIGAEHINKLIQVEGI
VTRVGEIKPFVSVAVFVCKDCGHEMIVPQKPYESLEKVKKCEQCGSKNIELDVNKSSF
VNFQSFRIQDRPETLKGGEMPRFIDGILLDDIVDVALPGDRVIVTGILRVVLEKREKTPIF
RKILEVNHIEPVSKEIQELEISPEEEQIIKELAKRKDIVDAIVDSIAPAIYGYKEVKKGIALAL
FGGVSRKLPDGTRLRGDIHVLLVGDPGVAKSQILRYVANLAPRAIYTSGKSSSAAGLTA
AAVRDEFTGGWVLEAGALVLADGGYALIDELDKMSDRDRSVIHEALEQQTISISKAGIT
ATLNARTTVIAAANPKQGRFNRMKNPFEQIDLPPTLLSRFDLIFVLIDEPDDKIDSEVAR
HILRVRRGESEVVAPKIPHEILRKYIAYARKNIHPVISEEAMEEIEKYYVRMRKSVKKTKG
EEEGIPPIPITARQLEALIRLSEAHARMRLSPIVTREDAREAIKLMEYTLKQIAMDETGQID
VTILELGQSARKLSKIEKILDIIEKLQKTSERGAHVNDILEEAKKAGIEKQEAREILEKLLEK
GQIYMPESGYYKTV
Predicted MW: 76.8kD

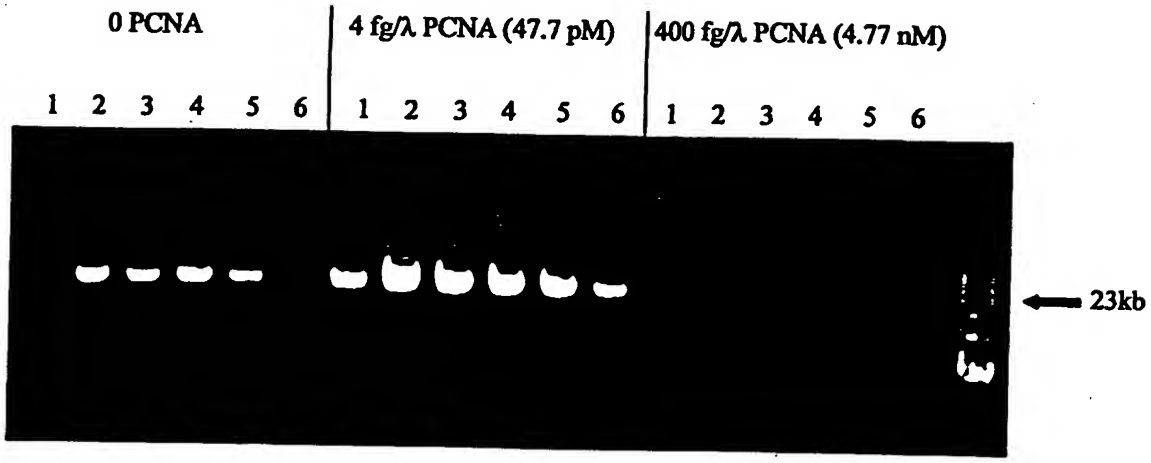
Figure 48. Nucleic acid sequence of *P. furiosus cdc6* gene

ATGAACGAAGGTGAACATCAAATAAAGCTTGACGAGCTATTCGAAAAGTTGCTCCG
AGCTAGGAAGATATTCAAAAACAAAGATGTCCTTAGGCATAGCTATACTCCCAAGG
ATCTACCTCACAGACATGAGCAAATAGAAACTCTCGCCCAAATTTTAGTACCAGTTC
TCAGAGGAGAAACTCCATCAAACATATTCGTTTATGGGAAGACTGGAACTGGAAAG
ACTGTAACTGTAAAATTTGTAACTGAAGAGCTGAAAAGAATATCTGAAAAATACAAC
ATTCCAGTTGATGTGATCTACATTAATTGTGAGATTGTCGATACTCACTATAGAGTT
CTTGCTAACATAGTTAACTACTTCAAAGATGAGACTGGGATTGAAGTTCCAATGGTA
GGTTGGCCTACCGATGAAGTTTACGCAAAGCTTAAGCAGGTTATAGATATGAAGGA
GAGGTTTGTGATAATTGTGTTGGATGAAATTGACAAGTTGGTAAAGAAGAGTGGTG
ATGAGGTTCTCTATTCATTAACAAGAATAAATACTGAACTTAAAAGGGCTAAAGTGA
GTGTAATTGGTATATCAAACGACCTTAAATTTAAAGAGTATCTAGATCCAAGAGTTC
TCTCAAGTTTGAGTGAGGAAGAGGTGGTATTCCCACCCTATGATGCAAATCAGCTT
AGGGATATACTGACCCAAAGAGCTGAAGAGGCCTTTTATCCTGGGGTTTTAGACGA
AGGTGTGATTCCCCTCTGTGCAGCATTAGCTGCTAGAGAGCATGGAGATGCAAGA
AAGGCACTTGACCTTCTAAGAGTTGCAGGGGAAATAGCGGAAAGAGAAGGGGCAA
GTAAAGTAACTGAAAAGCATGTTTGGAAAGCCCAGGAAAAGATTGAACAGGACATG
ATGGAGGAGGTAATAAAAACTCTACCCCTTCAGTCAAAAGTTCTCCTCTATGCCATA
GTTCTTTTGGACGAAAACGGCGATTTACCAGCAAATACTGGGGATGTTTACGCTGT
TTATAGGGAATTGTGCGAGTACATTGACTTGGAACCTCTCACCCAAAGAAGGATAA
GTGATCTAATTAATGAGCTTGACATGCTTGGAATAATAAATGCAAAAGTTGTTAGTA
AGGGGAGATATGGGAGGACAAAGGAAATAAGGCTTAACGTTACCTCATATAAGATA
AGAAATGTGCTGAGATATGATTACTCTATTCAGCCCCTCCTCACAATTTCCCTTAAG
AGTGAGCAGAGGAGGTTGATCTAA

Figure 49. Translated amino acid sequence of *P. furiosus* CDC6 protein

MNEGEHQIKLDELFEKLLRARKIFKNKDVLRHSYTPKDLPHRHEQIETLAQILVPVLRGE
TPSNIFVYGKTGTGKTVTVKFVTEELKRISEKYNIPVDVIYINCEIVDTHYRVLANIVNYFK
DETGIEVPMVGWPTDEVYAKLKQVIDMKERFVIIVLDEIDKLVKKSGDEVLYSLTRINTEL
KRAKVSVIGISNDLKFKEYLDPRVLSSLSEEEVVFPPYDANQLRDILTQRAEEAFYPGVL
DEGVIPLCAALAAREHGDARKALDLLRVAGEIAEREGASKVTEKHVWKAQEKIEQDMM
EEVIKTLPLQSKVLLYAIVLLDENGDLPANTGDVYAVYRELCEYIDLEPLTQRRISDLINE
LDMLGIINAKVVSKGRYGRTKEIRLNVTSYKIRNVLRYDYSIQPLLTISLKSEQRRLI

TaqPlus Long
23kb human β-globin



Concentration of FEN-1/λ RXN

1. 0
2. 460 fg (11.9 pM)
3. 920 fg (23.7 pM)
4. 4.6 pg (119 pM)
5. 46 pg (1.19 nM)
6. 460 pg (11.9 nM)

Figure 50

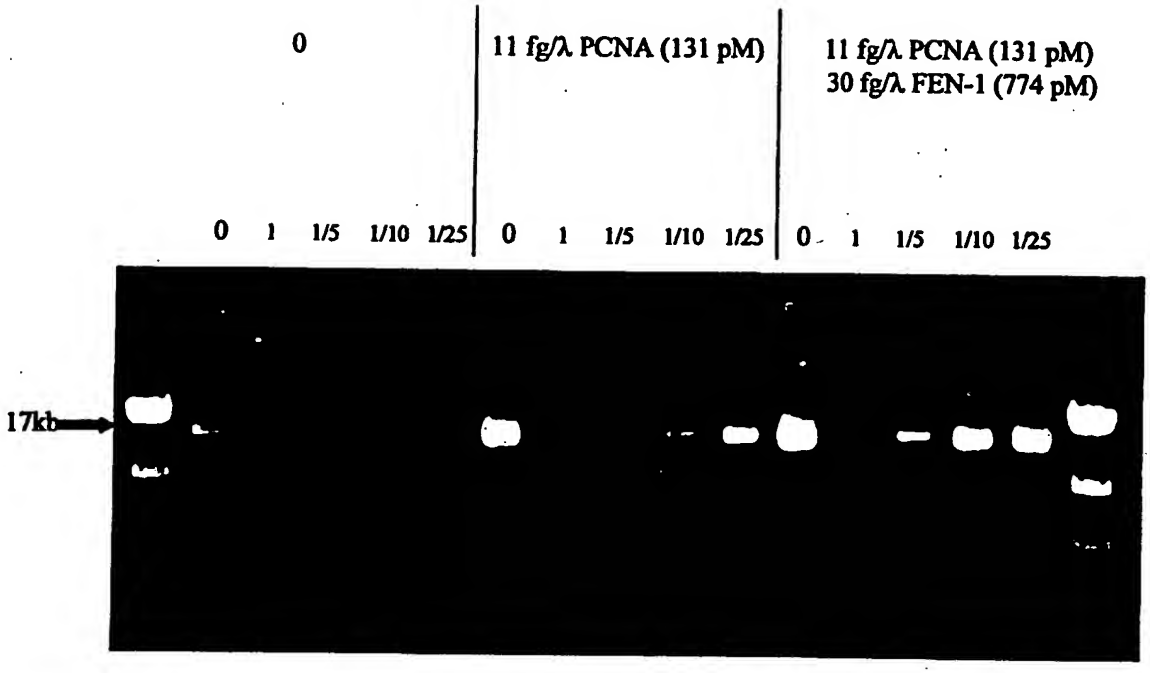
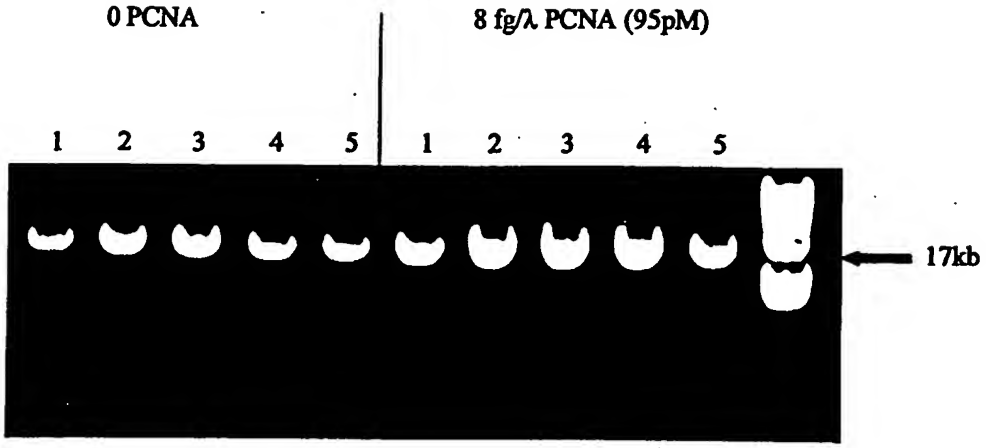

0
11 fg/λ PCNA (131 pM)
11 fg/λ PCNA (131 pM)
30 fg/λ FEN-1 (774 pM)
0 1 1/5 1/10 1/25 0 1 1/5 1/10 1/25 0 1 1/5 1/10 1/25
17kb

Figure 51

Pfu Turbo/β-globin



Concentration of FEN/λ PCR RXN

1. 0
2. 460 fg (11.9 pMl)
3. 920fg (23.7 pM)
4. 4.6pg (119 pM)
5. 46pg (1.19 nM)

FIGURE 52

TaqPlus Long/no PEF

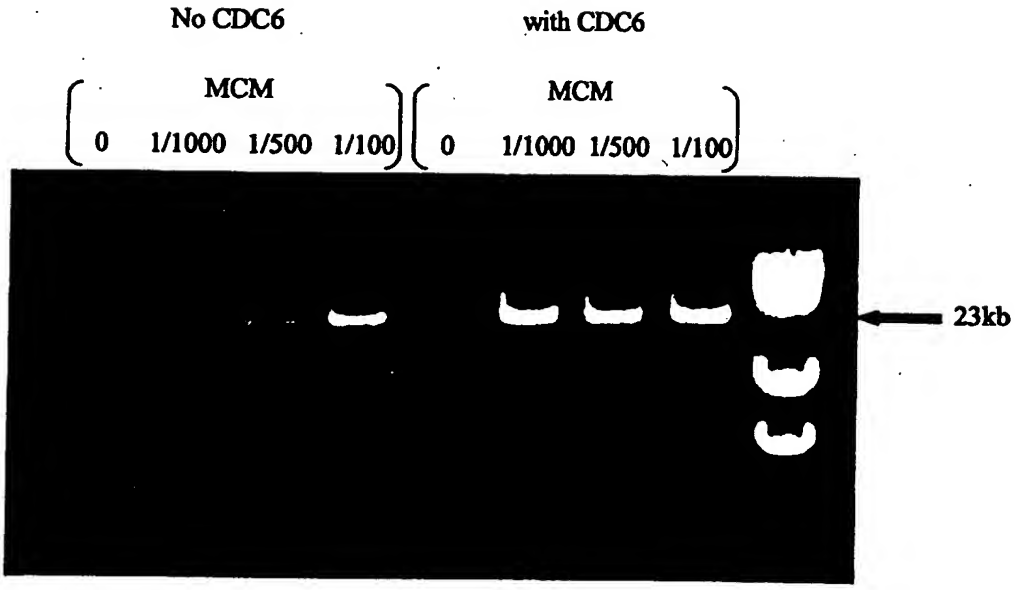


Figure 53

Figure 54

FIGURE 55

GACAGGGAGGAGATGATTGAGAGATTTGCAAACTTCCTTAGGGAGTATACAGACGA
AGATGGTAACCCAGTATACAGAGGTAAAATAACTGATTTACTTACAATAACACCCAA
GAGGTCTGTTGCAATAGACTGGATGCACCTAAATTCCTTTGACTCAGAGCTAGCTC
ATGAAGTTATAGAGAACCCCGAAGAAGGAATAAGTGCCGCAGAAGATGCAATCCAG
ATTGTATTACGAGAAGACTTCCAAAGAGAAGACGTGGGAAAAATACACGCAAGGTT
TTATAATTTGCCAGAAACCCTAATGGTCAAAGACATTGGGGCAGAGCACATCAACAA
GTTAATTCAAGTAGAAGGAATCGTGACGAGAGTAGGAGAAATTAAGCCCTTTGTCT
CTGTGGCAGTTTTCGTATGTAAGGACTGCGGTCATGAAATGATAGTGCCTCAAAAA
CCCTATGAAAGCCTTGAAAAAGTTAAGAAGTGCGAACAATGTGGAAGCAAAAATATA
GAACTAGATGTTAACAAGAGCTCCTTCGTAAACTTCCAGAGCTTTAGGATTCAGGAT
AGACCAGAAACCCTAAAAGGAGGAGAAATGCCAAGGTTTATCGATGGTATTCTGCT
TGATGACATAGTGGATGTAGCCCTCCCAGGAGACAGAGTTATTGTAACAGGAATTT
TGAGAGTCGTTCTTGAAAAGAGAGAGAAAACTCCAATATTTAGAAAAATCCTCGAGG
TAAATCACATTGAACCTGTTAGTAAAGAGATACAAGAATTAGAAATTTCTCCAGAAG
AGGAGCAGATAATAAAGGAGCTAGCAAAAAGAAAAGACATAGTAGATGCAATAGTT
GATTCAATAGCTCCTGCCATATATGGATACAAAGAAGTCAAGAAGGGAATAGCACTT
GCCCTGTTTGGAGGAGTTTCAAGAAAGTTACCTGATGGAACTAGGCTTAGAGGAGA
TATACATGTCCTCCTGGTCGGAGACCCAGGAGTTGCAAAGAGCCAGATTTTAAGGT
ATGTGGCAAACCTCGCTCCTAGGGCCATTTACACTTCAGGAAAAAGTAGTTCCGCA
GCAGGTCTTACTGCTGCAGCAGTTAGGGATGAGTTCACGGGAGGATGGGTTTTGG
AGGCGGGAGCTTTAGTCCTTGCAGATGGGGGTTATGCTCTAATCGACGAGCTCGA
CAAGATGAGCGACAGGGATAGGAGCGTGATACATGAAGCCTTAGAACAACAGACA
ATAAGCATTTCAAAAGCAGGGATTACGGCAACTCTAAACGCTAGAACTACAGTCATT
GCGGCTGCAAATCCGAAACAGGGAAGATTTAATAGAATGAAAAATCCATTCGAGCA
AATTGACCTTCCCCCTACACTTCTAAGTAGATTTGACCTAATATTTGTGTTAATTGAT
GAGCCCGATGACAAAATTGACAGTGAAGTTGCCAGACACATCTTAAGGGTCAGAAG
GGGAGAAAGTGAAGTCGTGGCCCCAAAAATACCTCATGAAATTCTAAGGAAGTACA
TCGCTTATGCAAGGAAGAATATTCATCCCGTTATAAGTGAAGAAGCTATGGAAGAGA
TAGAGAAGTACTATGTGAGAATGAGAAAGAGTGTAAAGAAGACAAAGGGAGAAGAA
GAGGGGATACCACCAATCCCAATAACAGCTAGACAGCTCGAGGCCCTCATTAGATT
AAGCGAAGCTCATGCAAGGATGAGGCTAAGCCCAATAGTAACAAGGGAAGATGCA
AGAGAAGCAATAAAACTGATGGAATACACGCTAAAGCAAATTGCAATGGATGAGAC
CGGGCAAATTGACGTGACAATTCTAGAATTAGGTCAGAGCGCAAGAAAGCTCAGTA
AAATAGAGAAAATACTGGATATCATTGAAAAGCTTCAGAAGACCAGCGAAAGAGGC
GCCCACGTTAATGATATCTTAGAAGAAGCAAAGAAAGCAGGAATAGAGAAGCAGGA
AGCAAGAGAAATCCTTGAAAAACTTTTGGAGAAGGGTCAAATATATATGCCAGAGA
GTGGTTACTACAAAACCGTCTGA (SEQ ID NO:)